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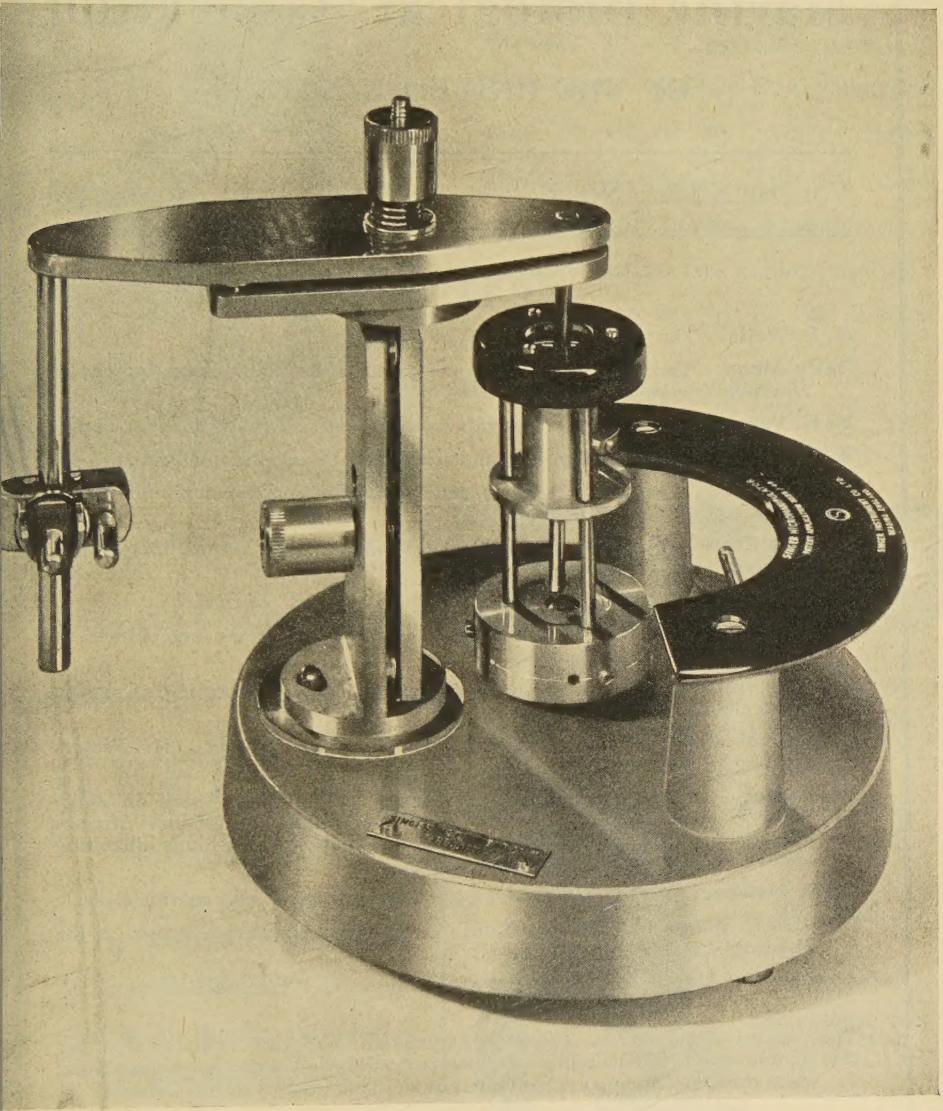

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The Multiplication of Vaccinia Virus in Tissue Cultures of Adult Rabbit Skin

By G. N. C. CRAWFORD AND F. K. SANDERS

(*From the Departments of Human Anatomy, and Zoology and Comparative Anatomy, Oxford*)

Summary

1. The optimum conditions for vaccinia virus multiplication in shavings of adult rabbit skin maintained in rocker flasks in a fluid medium have been investigated. Satisfactory virus multiplication occurred in 5 ml. of medium containing 40 per cent. rabbit serum, 40 per cent. phosphate-buffered saline, and 20 per cent. mouse embryo extract; an air atmosphere was used.

2. A growth curve for vaccinia virus grown *in vitro* under these conditions was obtained by estimating the virus content of cultured skin at known intervals after inoculation. The curve was essentially similar to that obtained by growth of vaccinia *in vivo*, and could be divided into three phases: (*a*) during the first few hours infectivity fell, (*b*) from 10 to 40 hours there was a rapid increase of infectivity, and (*c*) flattening of the curve started after 40 hours, although some increase of virus continued, until, at 80 hours, the concentration of virus present was at a maximum.

3. An attempt is made to correlate the multiplication of virus with the cytological changes known to occur in infected cells.

Introduction

At the present time most of our knowledge of virus multiplication comes from a study of bacterial viruses, since they can easily be freed from contaminating material and used to infect standardized suspensions of susceptible bacteria maintained in simple media. Consequently more is known of the actual process of virus synthesis with respect to these agents than with those infecting animal tissues. Growth of a bacterial virus within its host cell can roughly be divided into four phases: (1) a period of 'adsorption' and penetration into the cell; (2) a constant period, during which new virus material may be synthesized; (3) a period of rapid resumption of infectivity, followed by (4) a period during which virus is released, often by sudden lysis of the infected cells (Luria, 1950). It would be of interest to discover whether a similar growth cycle characterizes the multiplication of viruses in general, in particular such viruses as vaccinia and psittacosis, which approximate to small bacteria in size and chemical complexity (Smadel and Hoagland, 1942).

In the case of most animal viruses it is difficult even to approach the standardized conditions which are essential for a study of this sort. The source of virus is usually a crude suspension of ground-up infected tissue which contains much apart from active virus particles. Moreover, the tissue inoculated contains many kinds of cells differently situated as regards susceptibility and availability for infection; so the number of susceptible cells

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reached by the inoculum is almost always unknown. And, when experiments are conducted *in vivo*, the whole result may be complicated by nutritional and genetic factors, or by an immune response on the part of the host organism. However, in the case of vaccinia virus there is an opportunity of making an approximation to the standardized conditions used with bacterial viruses. The extra-cellular infective phase of this virus consists of brick-shaped particles roughly 236×200 mμ, which can be obtained fairly easily in purified form from a variety of infected tissues (Smadel and Hoagland, 1942). Moreover, virus multiplication can occur in association with fragments of various animal tissues suspended in serum or other nutrient fluids. Virus growth has thus been obtained in rabbit tissue with minced testis, kidney, spleen, cornea, or suspensions of Kupffer or mononuclear cells; minced whole chick embryos have also been used (see van Rooyen and Rhodes, 1948, for bibliography). Therefore, with purified virus and tissue cultivated *in vitro*, it might be possible to study virus growth under approximately standardized conditions.

The experiments described in this paper were undertaken with the object of exploring the possibilities of such a system, the first step being to obtain a picture of virus growth under tissue culture conditions and to compare it with that obtained with the same tissue *in vivo*. Rabbit skin was selected for investigation since virus multiplies readily to high titre in this tissue, causing the production of definite lesions. Also Medawar (1948) has shown that small fragments of rabbit skin can be maintained easily up to 8 days *in vitro*.

Virus

The virus used was the WR strain of vaccinia virus obtained through the courtesy of the Army Medical Department Research and Graduate School, Washington, D.C., U.S.A. This virus had had at least nine passages through the brains of mice before the present experiments were undertaken. A 10 per cent. suspension in saline of the infected brains from the ninth of these passages was inoculated into the shaved skin of the back and flank of a normal adult rabbit. On the third day a confluent vaccinial lesion had developed. The animal was then killed, and a suspension of purified elementary bodies of vaccinia virus was obtained from the skin by the technique of Craigie (1932). Briefly, this consisted of lightly scraping the surface of the infected skin into a pool of McIlvaine's buffered saline (pH 7·2), so that a suspension consisting of virus and cell debris was obtained. By centrifuging the latter differentially, an almost pure saline suspension of vaccinia elementary bodies could be obtained. Such a suspension formed the virus pool that was used throughout these experiments. It was kept stored at -70° C. in a carbon dioxide ice cabinet, samples being thawed out for use when required, and under these conditions its virus content did not alter significantly over long periods.

Estimation of virus content. The amount of virus present in suspensions was estimated by the inoculation of serial dilutions into the skin of adult

castrated male albino rabbits obtained from the Agricultural Research Council Field Station, Compton, Berks. They proved to be very susceptible to this strain of virus, sharp and consistent end-points being obtained. Tenfold dilutions of all material to be titrated were made up in Simms and Sanders (1942) saline X. 6 (a phosphate bicarbonate buffered saline of pH 7·6, which was also used as a constituent of the culture medium). 0·1 ml. quantities of each dilution were injected intracutaneously into the shaved back and flanks of the rabbit, five injections of each dilution being made. The rabbit was examined for lesions on the second to fifth days after inoculation. That dilution of the suspension which, when injected in these circumstances, caused a lesion in 50 per cent. of cases (that is contained one 'infective dose' (ID_{50}) per 0·1 ml.) was calculated by the method of Reed and Muench (1938). Such titrations are accurate to within 0·2 of the interval between successive dilutions. In the case of tenfold dilutions the limit of accuracy is thus about $\pm 10^{0.2}$. For example, it is probably not significant that in one experiment the virus content of skin grown in 10 ml. of ultrafiltrate-containing medium is about double that grown in 5 ml. of the serum-containing medium (see Table 2).

The virus pool described above and used throughout these experiments contained $10^{6.6}$ ID_{50} per ml.

Growth of Vaccinia Virus *In Vivo*

In order to have a standard by which to judge the growth of virus in tissue cultures of skin, an attempt was made to obtain a curve showing the growth of virus in the skin of an adult rabbit.

The skin of the back and flanks of a normal adult albino male rabbit was shaved and twenty-four separate symmetrically placed 0·1 ml. intradermal inoculations, each containing approximately 10 ID_{50} of virus, were made. At 0, $1\frac{1}{2}$, 3, 6, 12, 24, 48, and 72 hours after injection, three pieces of infected skin were shaved off with a safety razor blade, as little connective tissue as possible being removed with the epidermis. The three pieces of skin removed at each time interval were stored together at $-70°$ C. until all had been so harvested. Each set of three pieces of skin was then weighed on a torsion balance and ground in sufficient saline to make a 10 per cent. suspension. It was found that over 80 per cent. of the tissue went into suspension, the residue being presumably mainly collagen. The virus content of the tissue was then estimated, and expressed as ID_{50} per mg. of tissue. The values obtained at each time chosen are plotted against time in fig. 1. The method of assay adopted does not allow of a particularly accurate estimation of the small amount of virus present in the skin during the first few hours following infection. However, it appears as though there is a phase of increase in infectivity extending over the period from 24 to 72 hours after infection. The maximum virus titre is first reached after about 80 hours. This compares closely with the result obtained by Widelock (1938).

Growth of Vaccinia Virus *In Vitro*

The basic skin culture technique used in these experiments has been described by Medawar (1947 and 1948). From adult rabbits he shaved off thin pieces of lightly vaselined skin which were then floated on the surface of medium contained in conical flasks, kept rocking in an incubator at 37° C. The most favourable medium consisted of serum, together with adult tissue extracts and some glucose. Cultures were made either anaerobically, in the

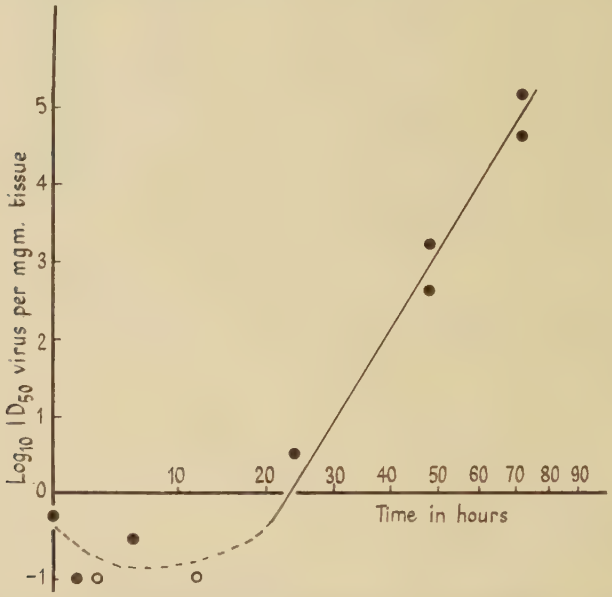

Fig. 1. Graph showing the changes of vaccinia virus concentration in infected normal adult rabbit skin *in vivo*. Unfilled circles—no virus recoverable from tissue.

presence of air, or at a high oxygen pressure. Under all three conditions the skin survived for periods up to 8 days without renewal of the medium. Under anaerobic conditions, however, neither cell migration nor mitoses occurred. In the other two cases the epithelium of the cultured skin fragments migrated freely around the dermis, so that the fragment eventually became encysted. Epithelial migration, however, occurred more readily in an atmosphere of air, while mitoses were more frequent when a high oxygen pressure was used.

With this culture technique as a starting-point, it was necessary to discover those conditions most favourable for vaccinial multiplication in cultured skin. The factors first investigated were (1) the composition of the medium, (2) the composition of the gas phase, (3) the volume of the medium in the flask, (4) the mode whereby the skin could be infected.

Method of obtaining and culturing skin. Skin was obtained from the outer surface of the ear of normal adult rabbits. The region was shaved, rinsed with 1·5 per cent. cetavlon followed by 75 per cent. alcohol, which was

allowed to dry. 0·1 ml. intradermal inoculations were then made of saline, which, if necessary, contained vaccinia virus. Each injection caused the formation of a raised bleb of skin about 7 mm. in diameter, which was then shaved off with a safety razor blade. Care was taken to make the shavings as thin as possible; each one weighed approximately 20 mg. After washing in a large volume of solution X. 6, four skin shavings were transferred to each of a number of sterile 500 ml. conical flasks fitted with flask heads for gas perfusion (Medawar, 1948). In later experiments each flask contained 5 ml. of tissue culture medium, which consisted of normal adult rabbit serum, Simms' solution (X. 6), and mouse embryo extract. The latter was prepared by mincing 14–18-day old mouse foetuses with scissors in an equal volume of Simms' solution and incubating the resulting suspension at 37° C. for 1 hour. The suspension was then centrifuged at 2,750 r.p.m. for 10 minutes and the supernatant stored at −70° C. over carbon dioxide ice until required. Streptomycin was added to the culture medium in all the experiments to give a final concentration of 2 mg. per ml. and was in every case successful in preventing bacterial contamination. The flasks were incubated at 37° C. on a continuously rocking platform, an angle of about 15 degrees on each side of the horizontal being achieved with each excursion. The speed of rocking was approximately 4 times per minute. In most experiments each flask contained 5 ml. of medium, and this did not cover the whole of the bottom of the flask when the rocking platform was fully tilted. The pieces of tissue thus tended to be washed intermittently by the medium as in roller-tube cultures.

In some experiments it was necessary to culture the infected skin at a high oxygen pressure. This was attained by perfusing the flask with a 95 per cent. oxygen/5 per cent. carbon dioxide mixture for 10 minutes daily, and then sealing off the flask head.

Effect of medium on vaccinial growth

(1) Experiments with another strain of virus had previously shown that vaccinia grew well in the presence of serum diluted with Simms' solution (X. 6). The following experiment tested the effect of adding embryo extract to the medium, and of high oxygen tension.

Skin shavings were obtained from the rabbit's ear as described above (p. 122). In this case, however, each injection of saline into the ear skin contained about 40 ID_{50} of vaccinia virus –that is, about 2 ID_{50} per mg. of skin shaved off. After washing in solution X. 6 for 20 minutes, four shavings were transferred to each of two pairs of conical flasks containing 5 ml. of medium. In one pair of flasks the medium consisted of equal parts of serum and saline only, while to the other pair embryo extract had also been added at a final concentration of 20 per cent. One flask of each pair was gassed intermittently (see above) with 95 per cent. oxygen and 5 per cent. carbon dioxide (see Table 1).

All flasks were incubated at 37° C. for 72 hours, at the end of which time the skin was removed, washed, and the quantity of virus present in each case

estimated by the method already described. The concentration of virus attained in each of the four sets of skin fragments is shown in Table 1.

TABLE 1. *Effect of (1) culture medium, and (2) flask atmosphere on the multiplication of vaccinia virus in skin cultures. Figures = ID_{50} of virus present per mg. tissue harvested*

	Composition of flask atmosphere	
Composition of medium	*Air*	*95 per cent. O_2; 5 per cent. CO_2*
Serum-saline (1:1) no embryo-extract	400	20
Serum-saline (1:1) +20 per cent. embryo-extract	1,580	16

Culture of skin in an atmosphere of air resulted in the production of 25–100 times as much virus as when a high oxygen pressure was used. Addition of embryo extract to the basic medium did not greatly affect the final virus titre in the presence of a high oxygen pressure. However, in an atmosphere of air, embryo-extract increased the virus yield approximately fourfold.

(2) Experiments were also made to test whether virus yield was affected by the volume of medium present, or by replacing its serum content by ox serum ultra-filtrate (Simms and Sanders, 1942).

Infected skin shavings were obtained exactly as described in the previous experiment, the dosage of virus per mg. of skin being the same. Three pairs of flasks were set up, each containing four pieces of skin. One pair contained $2\frac{1}{2}$ ml., one 5 ml., and one 10 ml. of medium per flask. Within each pair, the medium in one flask consisted of 40 per cent. serum, 40 per cent. saline, and 20 per cent. mouse embryo extract; the medium in the other flask consisted of 20 per cent. ox serum ultrafiltrate, 60 per cent. saline, and 20 per cent. embryo extract. All flasks were incubated at 37° C. for 72 hours, at the end of which time the skin was removed, washed, and its virus content determined. The result of this experiment is shown in Table 2.

TABLE 2. *Effect of (1) volume, and (2) presence of serum ultrafiltrate in culture medium on the multiplication of vaccinia virus in skin cultures. Figures = ID_{50} of virus present per mg. of tissue harvested*

	Volume of medium in ml.		
Composition of medium	*2·5*	*5*	*10*
Serum (40 per cent.) Saline (40 per cent.) Embryo-extract (20 per cent.)	25	3,200	32
Serum ultrafiltrate (20 per cent.) Saline (60 per cent.) Embryo-extract (20 per cent.)	320	3	6,300

When the medium contained serum, the concentration of virus in the skin grown in the flask containing 5 ml. was about 100 times as high as when the flask contained either 10 or $2\frac{1}{2}$ ml. The maximum concentration of virus in the skin was not much increased by substituting ultrafiltrate for serum. On the other hand, the yield of virus was higher when 10 ml. of the ultrafiltrate containing medium was used as compared with 5 or $2\frac{1}{2}$ ml. Undue significance should not be attached to the low virus titre attained when the skin was grown in 5 ml. of the ultrafiltrate containing medium. This rather anomalous result was based on a single experiment, which, in view of the fact that the use of ultrafiltrate rather than serum did not greatly increase the growth of vaccinia in the skin, seemed scarcely worth repeating. Five ml. of a similarly constituted serum medium were used routinely in subsequent experiments.

(3) Finally, an experiment was performed in order to determine that method of infecting the skin with virus, which resulted in the highest virus titre.

0·1 ml. injections of solution X. 6 alone were made into the skin of a rabbit's ear, and the blebs of skin were shaved off as previously described. Four of the skin shavings so obtained were then infected by floating them for 15 minutes in 2 ml. of solution X. 6 containing virus at a concentration of 400 ID_{50} per ml.; four more were infected by floating them for 15 minutes in 2 ml. of normal rabbit serum, containing virus at the same concentration. Another four skin shavings were washed in saline for 30 minutes, after which each was injected *in vitro* with 40 ID_{50} of virus. In the case of four other pieces of skin, virus was added to the medium in the flask in which they were subsequently cultured at a concentration of 400 ID_{50} per ml. Finally, as a control, four 0·1 ml. intradermal injections of 40 ID_{50} were then made *in vivo* into the skin of the ear and the infected skin blebs shaved off.

The batches of skin infected by these different methods were cultured in separate flasks in 5 ml. of medium consisting of 40 per cent. normal rabbit serum, 40 per cent. saline, and 20 per cent. embryo extract; all flasks were incubated for 72 hours, when the pieces of skin were removed, washed, and their virus content determined.

TABLE 3. *Effect of method of inoculation on multiplication of vaccinia virus in skin cultures*

Method of inoculation	*Virus present after 72 hours culture* (LD_{50}/*mg.*)
Skin injected *in vivo*	320,000
Skin cultured in medium containing virus	320,000
Skin floated on saline + virus	4,000
Skin injected *in vitro*	$\leqslant$ 630
Skin floated on serum + virus	$\leqslant$ 3

The result of this experiment is given in Table 3. It is evident that the best yield of virus is obtained when the latter is infected either by injecting the skin *in vivo* or by adding virus to the culture medium. Any other mode of infection appears to be less effective. When the skin is exposed to infection by floating it on virus suspended in serum for 15 minutes very little, if any, virus is produced, a result which was confirmed by a second experiment. The routine method of infection adopted in subsequent experiments has thus been inoculation of virus into the skin before its removal from the rabbit.

Growth curve of vaccinia in skin maintained in vitro

With the above technique of infection, and the skin so infected being cultured in 5 ml. of the serum saline embryo-extract medium, an attempt was made to construct a growth curve for virus under these conditions.

Pieces of skin, in groups of three, were removed from the flasks at intervals from 1½ to 96 hours after setting up the cultures. After thorough washing in Simms' solution they were stored at −70° C. until required. When all the tissue had been harvested, the pieces of skin were ground in batches to give 10 per cent. suspensions in saline; the entire tissue went into suspension. The amount of virus present in the tissue after each time interval was then estimated as already described (p. 120). The values obtained, expressed as ID_{50} per mg. of skin, are plotted against time in fig. 2. It will be seen that the growth of virus in skin maintained *in vitro* follows a pattern very similar to that obtained when the virus is grown in the intact skin of the animal (see fig. 1). During the first few hours there is a decline in the amount of infectivity present; after about 10 hours, however, the infectivity of the tissue begins to rise once more. Flattening of the curve starts rather before 48 hours, but the maximal titre is not reached until the skin has been in culture for about 80 hours. There seems to be little difference between the final titres attained, whether the virus is grown in skin in tissue culture by this method or on the animal. As, under the conditions used, every susceptible cell in the piece of skin is unlikely to have been infected at the outset, the curve of fig. 2 probably does not represent a one-step growth cycle of the type described for bacterial viruses (Delbrück, 1946). Virus liberated from the cells at the end of the first cycle is free to infect further cells, so that the whole curve probably represents a summation of several small cycles. The time before infectivity begins to come back, however, may indicate the length of the constant phase of the first cycle, and the resurgence of infectivity which begins at 10 to 12 hours after infection may be the first crop of new virus particles produced. The cycle, however, must differ widely from that of the bacterial viruses, particularly in time relations. The constant period of bacterial virus T_2 on *Escherichia coli* grown in broth is roughly 20 minutes. That of vaccinia virus seems to last about 10 hours. However, this need not mean that a quite different method of virus reproduction is involved. If virus synthesis depends on the activity of the enzymes of the host cell, energy must be supplied by the host cell's respiratory mechanism. The respiratory

rate of mammalian skin is approximately one-twenty-fifth of that of rapidly dividing *E. coli* (Krebs, 1934). The constant period of vaccinia virus is about 30 times as long as that of the bacterial virus. The difference is thus roughly the same as that in the respiratory rates of the cells concerned.

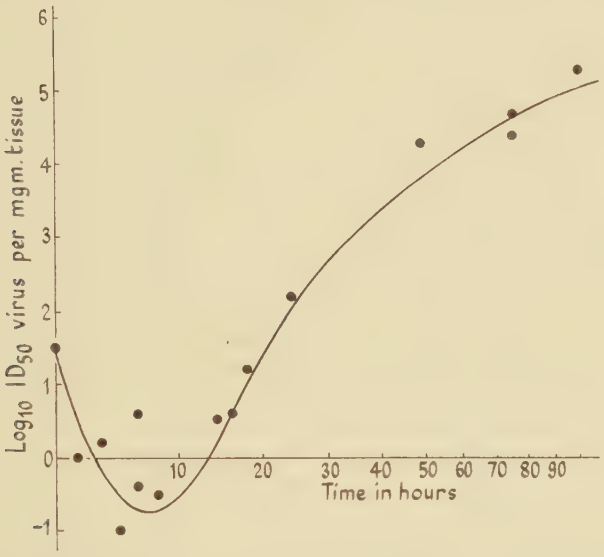

FIG. 2. Graph showing the changes of virus concentration in vaccinia-infected shavings of adult rabbit skin maintained *in vitro* in a saline serum embryo-extract medium.

DISCUSSION

When the growth of vaccinia virus in skin maintained *in vitro* is measured by the amount of infectivity recoverable from the tissue at intervals after inoculation, a curve is obtained in which it is possible to distinguish phases analogous to those described for viruses such as influenza (Hoyle, 1948, 1950) and the bacterial viruses (Luria, 1950). The growth curve of vaccinia in rabbit skin maintained *in vitro* may be divided into: (1) a period of falling infectivity; (2) a period during which infectivity remains at a low level, followed by (3) a period of rapid resumption of infectivity. It is suggested that the first two of these periods may correspond respectively to the 'adsorptive' and 'constant' phases in the growth cycle of bacterial viruses, and it is possible that there may be a fundamental similarity in the mode of reproduction of the two types of virus. Further insight into this process may be gained when the cytological changes which take place in vaccinia-infected cells are considered in relation to the growth curve described above (fig. 2).

The infection of susceptible cells by vaccinia virus is accompanied by the appearance of well-defined and characteristic inclusion bodies in the cytoplasm, long recognized as diagnostic for this disease. The type of inclusion seen, however, varies with different phases of the infectious process; for

example, the classical eosinophilic inclusions first noted by Guarneri (1892) do not appear until late in the disease. The course of development of vaccinia inclusions, however, has been described quantitatively by Bland and Robinow (1939), who studied the cytology of hanging drop tissue cultures of rabbit cornea at intervals after the inoculation of such cultures with a large dose of

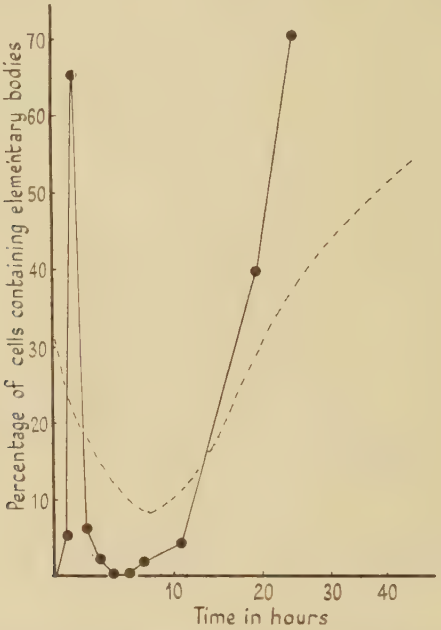

FIG. 3. Comparison of the percentage of cells containing vaccinia elementary bodies at various times after infection, with the growth curve of vaccinia virus in skin maintained *in vitro*. Solid line, percentage of cells containing elementary bodies (from data of Bland and Robinow); dotted line, vaccinia growth curve (present data).

virus, and noted the appearance within the cytoplasm of infected cells of three distinct types of abnormal structures:

1. *Elementary bodies*. These are small, basiphil, Feulgen-positive particles about 0·25 μ in diameter, which correspond in size and staining reaction with the elementary or Paschen bodies thought to be the infective units of this virus.
2. '*Homogeneous*' *bodies*. These are strongly basiphil and also Feulgen-positive, but are larger than elementary bodies, being up to 5 μ in diameter.
3. '*Networks*'. These are acidophil condensations or clouds in the cytoplasm, and were usually found in association with elementary bodies.

Bland and Robinow made careful counts of these different inclusions at intervals from 1½ hours to 24 hours after inoculation (all the diagrams given

here of types of inclusions present are based upon their data). Fig. 3, for instance, shows the percentage of cells containing elementary bodies in their tissue cultures, plotted against time since inoculation. It will be seen that at the outset there is a marked rise in the proportion of cells containing elementary bodies, a maximum being reached after about 1½ hours. It may be conjectured that this phase represents the initial penetration of virus into the cells. From 1½ hours onwards, however, there is an equally spectacular fall in the proportion of cells containing elementary bodies, so that after 3 to 6 hours virtually no cells can be found which contain them. Starting at about 10 hours, however, there is a second increase in the proportion of cells containing elementary bodies. The increase this time is maintained, so that 24 hours after inoculation they are to be found in over 70 per cent. of the infected cells. The dotted line in fig. 3 shows the growth curve of vaccinia virus in tissue cultures of rabbit skin obtained during the present investigation. While the results obtained with our method cannot be directly related to those obtained by Bland and Robinow using a different tissue-culture system, a comparison of the data obtained in the two cases gives rise to an interesting hypothesis with regard to the possible mode of multiplication of vaccinia virus. Fig. 3 shows that the initial rise in the proportion of cells containing elementary bodies occurs at a time when the infectivity of the culture may actually be falling. Only in the later stage is a rise in the number of elementary bodies accompanied by a corresponding rise in infectivity.

Fig. 4 shows the situation with regard to the second type of inclusion—the 'homogeneous' bodies. Here again the initial stage of infection is accompanied by the appearance of large numbers of homogeneous bodies, which occur at this stage in association with elementary bodies in large numbers of cells. The subsequent decline in the proportion of cells containing elementary bodies is accompanied, or perhaps followed, by a like fall in the number of cells containing homogeneous bodies. All this probably occurs while the infectivity of the culture is growing less. The secondary rise after about 10 hours both of infectivity and in the proportion of cells with elementary bodies is not accompanied by a similar increase in the number of homogeneous bodies. The number of cells with this kind of inclusion remains low for the rest of the period observed.

The number of 'networks' fluctuates in yet another way (fig. 5). At 1½ hours the number of networks is small. An increase in their number follows immediately, so that 2 to 3 hours after infection about 10 per cent. of the cells contain them. There is no rise and fall in the number of cells containing this inclusion at the start of infection, as there was with the other two types. From 2–3 until 10 hours after infection the number of networks remains approximately the same, or may even fall slightly. From 10 hours onwards, however, there is sudden increase in their number, so that at 24 hours over 70 per cent. of the cells have networks within them.

Elementary bodies thus appear to be associated with networks at the end of the cycle of infection, when infectivity is rising, and with homogeneous

bodies at its beginning when infectivity is falling. There is at present a great deal of evidence to identify the elementary bodies with the infectious unit of the virus (Smadel and Hoagland, 1942). It is thus possible that the initial stage of infection, which is characterized by the appearance of large numbers of elementary bodies within the cells and readily recoverable infectivity, is succeeded by a phase during which there is a change of the elementary

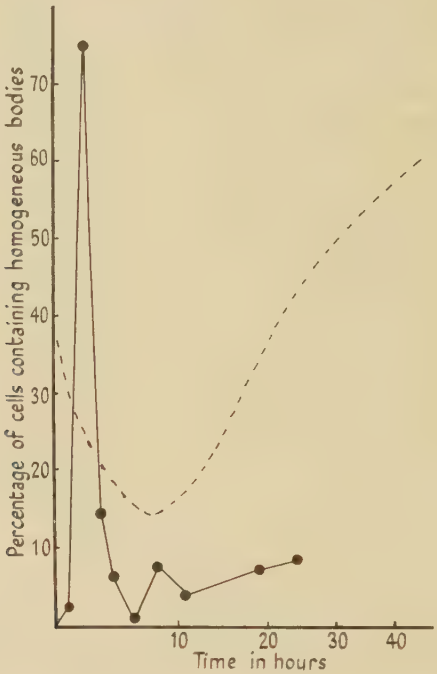

FIG. 4. Comparison of the percentage of cells containing 'homogeneous bodies' at various times after vaccinia infection, with the growth curve of vaccinia virus in skin maintained *in vitro*. Solid line, percentage of cells containing homogeneous bodies (from data of Bland and Robinow); dotted line, vaccinia growth curve (present data).

bodies into other units which are non-infective. The apparent disappearance of the Feulgen-positive particles at this time suggests that this change is accompanied by a loss of desoxypentose nucleic acid from the elementary bodies. Furthermore, the larger homogeneous bodies, which are themselves basiphil and Feulgen-positive, appear during this stage. They seem to be formed while the elementary bodies are disappearing, and Bland and Robinow's data suggest that they survive the virus particles. It is thus possible that part, at least, of the desoxypentose nucleic acid released from the virus particles during their breakdown is incorporated in the homogeneous bodies.

The reappearance of infectivity after 10 hours is again associated with the presence of elementary bodies. Thus during the latent period of 6–8 hours preceding this change, virus multiplication (as measured by infectivity) is not

evident, and the alteration of virus occurring at this stage may be a necessary preliminary to its multiplication. It is of course possible that actual formation of a 'precursor' or 'incomplete' form of the virus takes place during the latter part of this phase. The precursor may then increase in complexity, regaining, for example, its normal desoxypentose-nucleic acid content, and at the same time its infectivity.

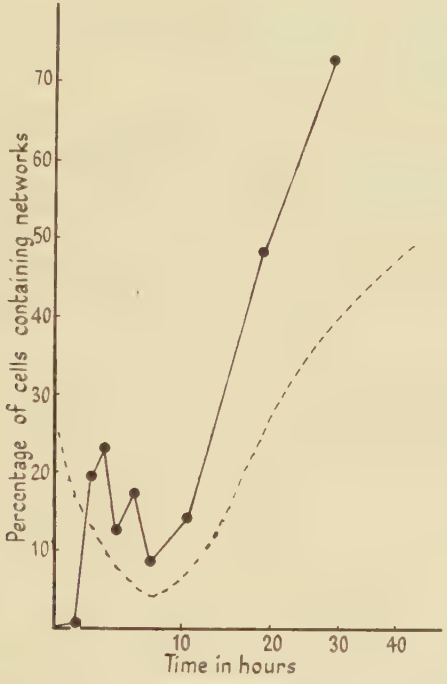

FIG. 5. Comparison of the percentage of cells containing 'networks' at various times following vaccinia infection, with the growth curve of vaccinia virus in skin maintained *in vitro*. Solid line, percentage of cells containing 'networks' (from data of Bland and Robinow); dotted line, vaccinia growth curve (present data).

That the acidophil networks are associated with virus multiplication is suggested by the fact that they increase in number just before the resumption of infectivity takes place. It is clear, though, that they do not represent the virus itself. They may, however, be derived from the virus particles, or, alternatively, they may arise within the cells as by-products of virus synthesis. In either case it is tempting to suggest an association with the soluble antigens, virus-specific but separable from the virus particles, known to be formed in infected tissues during virus multiplication (Smadel and Hoagland, 1942).

It cannot be pretended that the above represents more than a very tentative account of the possible growth cycle of vaccinia virus. It is clear, however, that tissue-culture methods have made it possible to approach this problem, and that further studies with the system we have used may make it possible to elucidate the cycle in more detail.

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Experiments with P^{32} and I^{131} on Species of *Helix*, *Arion*, and *Agriolimax*

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Summary

If *Helix aspersa*, *H. pomatia*, *Arion hortensis*, and *Agriolimax agrestis* be fed on a diet which contains P^{32}, autoradiographs show that the isotope is taken up by the digestive and lime cells of the digestive gland. From the former most of it passes to the haemocoel, though some is retained for immediate metabolic activities; in the lime cells it is stored in calcium spherules. A very small amount of the tracer enters the body through the wall of the oesophagus, and more through the intestine, this site of diffusion being most pronounced directly after hibernation.

The P^{32} in the haemocoel is dispersed to all tissues: all of them take up a little; in some it becomes concentrated. Concentrations appear in the nerve ring, the mucous and salivary glands, the odontophore and certain cells of the mantle.

In the nervous system deposits are heavy around the fibres and slight in the cytoplasm of the cells; they indicate a compound, soluble in alcohol, which may be phospholipine, associated with medullated nerves.

The phosphorus in mucous cells, most pronounced in the pedal and salivary glands, may be combined with the calcium which stabilizes mucus and prevents its rapid dispersal.

The incorporation of isotope into the developing tooth of the radula indicates the relative activity of the basoblasts and cuspidoblasts: in early development of a tooth the basoblast secretes more actively, but as it becomes effete secretion by the cuspidoblast is accelerated. When the tooth is liberated from the latter there is no further addition to its substance.

Phosphorus deposits in the mantle are in the calcium cells which secrete the shell. Here, as in the lime cells, and around certain blood-vessels, excess may be stored as calcium phosphate; reserves in the digestive gland are the largest.

Amoebocytes concerned with the regeneration of the shell of *Helix pomatia* and *H. aspersa* carry the tracer element, and some of it is deposited in the shell. Also in the slug the tracer is transported by amoebocytes.

Radioactive iodine in the lumen of the gut is taken up most readily by digestive cells; some enters the lime cells. Only in sparing quantities does this isotope pass from the gland to the rest of the body, and this entry is presumably associated with ionic exchange. It is not accumulated in any cell, except in the kidney whence it is excreted; it leaves the digestive cells to pass from the body with the faeces.

Introduction

THE use of radioactive isotopes has opened up a new approach to the study of intermediate metabolism. It allows extremely small concentrations of certain elements to be traced through the tissues of an organism. These may be taken up with the food of an animal: and then it is possible to find the site of their diffusion or absorption through the wall of the gut, the

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means by which they are transported, their localization, and, finally, the site of their excretion. The investigation of such a problem may be carried out by means of the Geiger–Müller counting apparatus, though the more detailed histological study involves the use of autoradiographs. The autograph records the radiations from the isotope on a photographic emulsion and so allows its concentration within specific cells to be located. This method has been employed in studying the metabolism of certain arthropods: Lindsay and Craig (1942) investigated the distribution of P^{32} in certain stages of the life-history of *Galleria melonella*, *Tenebrio molitor*, *Blatella germanica*, and *Thermobia domestica*, and Wheeler (1947) the iodine metabolism of *Drosophila gibberosa*. For the detection of comparatively small quantities of radioactive isotopes in the organs of small invertebrates the photographic technique is the more precise. If the Geiger–Müller counter be employed organs must be dissected out, brought into solution, and the activity of the solution recorded. Now the animals may be too small for accurate microdissection, which should guard against leakage of protoplasm, and hence isotope, from the tissues; moreover, in dissection there is often an inevitable loss of body-fluids, and the contamination of organs with blood.

Autoradiograph technique has been improved in recent years by the manufacture of very fine-grained stripping film such as that supplied by Kodak, Ltd. In this the sensitive emulsion is exceptionally thin and is coated on top of a gelatine layer which adheres to a glass plate; the gelatine gives sufficient robustness to permit handling. During the preparation of the autograph this double layer is stripped from the glass, and the emulsion is brought into intimate contact with a thin section (5–10μ) which contains radioactive material. After an exposure which may vary from 2 to 14 days, as in the present investigation, or may be extended to twice the half-life of the isotope, the film, adhering to the slide, is developed in amidol developer. Normally the autoradiograph and section remain superimposed, which gives the advantage of automatic alinement; the tissue may be stained with haemalum, and differentiation in 1 per cent. HCl takes the stain from the gelatine. If desired, however, the autograph may be floated off the sections and on to a clean slide (warm water may be used for this); the sections on the original slide may be kept for reference, stained, and mounted in the usual way.

In the present work the radioactive isotopes were supplied by the Isotope Division of the Atomic Energy Research Establishment, Harwell. The phosphorus was administered as sodium dihydrogen phosphate solution, and the iodine as sodium iodide; the activity of each was 200μ C. per millilitre.

Helix aspersa (Müller), *H. pomatia* L., *Arion hortensis* Férussac, and *Agriolimax agrestis* (L.), which were used for experiments, ate lettuce on to which had been evaporated the radioactive isotope, either P^{32} or I^{131}. A dose which ranged from $\frac{1}{2}$ 5μ C. was evenly distributed over the surface of a small piece of leaf, and the animals had from one to seven meals, each one at night. Each animal was isolated in a glass capsule covered with damp muslin; at such a high humidity it was active and ate well. The total consumption of isotope

was of the order of 4μ C. per gram of body weight; not all of this entered the tissues, for some was lost with the undigested plant tissue in the faeces.

The experimental animals were fixed directly after feeding on the contaminated lettuce; or they were taken from this and put on uncontaminated leaves for one to several days before being fixed. A number were kept alive for some weeks to see whether the radiations had any injurious effects, and these animals were not used further. The dose did not appear to harm them. Even on occasions when the dose was doubled the radiations did no apparent harm, for the individuals remained active, and growth by mitotic divisions could be seen on microexamination.

The animals which ate lettuce with I^{131} were fixed in absolute alcohol. Although for histological detail this is a poor fixative it has the advantage of producing good autoradiographs. Other fixatives are apt to inhibit the photographic process so that the part of the film overlying the section may resist development. Moreover, when an iodide is present in the tissues, fixatives containing mercury salts—Susa, Helly, Zenker—must be avoided, for the subsequent treatment with iodine to remove mercury deposits would also remove iodine compounds from the tissues. Such fixatives have been reserved for studying histological details only; these are needed to check the results of the autoradiographs. After eating P^{32} the animals were treated in one of two ways: some were fixed in absolute alcohol and paraffin sections were cut; others were fixed in 10 per cent. formaldehyde and after gelatine embedding the tissues were frozen and sectioned. The two processes are necessary since after the first one only phosphoproteins will be retained, whilst with the second only the P^{32} present as water soluble phosphorus is lost and phospholipines, phosphoproteins, hexose phosphates, phosphoglyceric acid, and adenyltriphosphoric acid will be retained. It is essential that the frozen sections be cut as thin as possible. They should not exceed 10μ, since, when film stripping is floated on to thicker sections, air is likely to be trapped around the edge of each, between the film and the slide, with the effect of warping. Moreover, the thicker the section the more diffuse is the autoradiograph. After both alcohol and formaldehyde it is essential that the sections be washed well in distilled water before the film is applied.

Site of Absorption of P^{32} from the Gut

When P^{32} is eaten by a slug or snail, autoradiographs show that the phosphorus passes into the cells of the digestive gland in abundance. The digestive cells of the gland are more numerous than the lime cells, and produce enzymes (Krijgsman, 1925), which are secreted into the gut; they also take up the products of digestion, and store glycogen and fat; their waste material is passed into the lumen of the digestive gland and voided with the faeces. The lime cells are concentrated in the crypts of the tubules. They are shorter than the digestive cells, pyramidal in shape, and each has a broad base of 50μ or more bordering the haemocoel. The cytoplasm is dense and vacuolated, the vacuoles containing spherules of calcium. No investigation has proved that these

spherules absorb their calcium directly from the gut; it may be brought to them by amoebocytes (Wagge, 1948), or they may obtain it from the blood. Heaysman (1951) states that in *Nucella lapillus* and *Helix pomatia* calcium is taken in through the wall of the gut and transported by the blood to the digestive gland. This, at least for *Helix*, may be one route by which calcium reaches the lime cells, for in this pulmonate there is a diffusion of some ions through the crop and intestine. However, I have been unable to find sufficient

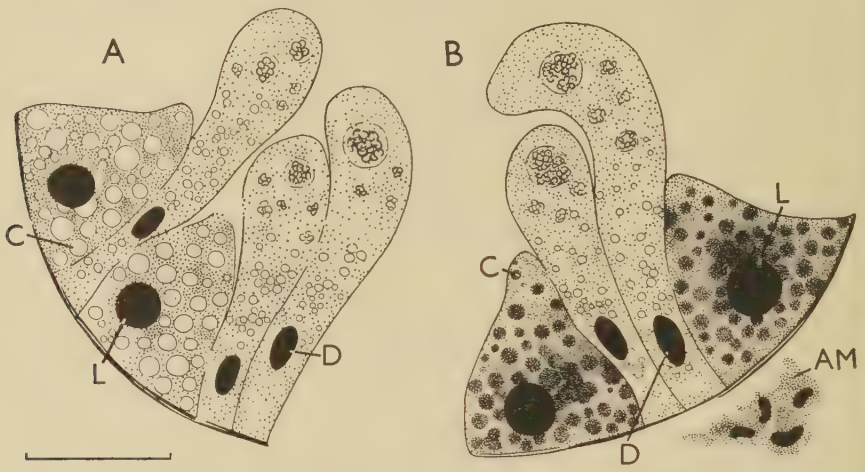

FIG. 1. Part of transverse sections through the digestive gland of *Arion hortensis* to show the distribution of P^{32}. Stippling represents superimposed autoradiographs. Slugs were fixed in absolute alcohol after feeding on lettuce with P^{32}: A, 9 hours after commencing to feed; B, 48 hours after commencing to feed. AM, amoebocytes; C, calcium spherule; D, nucleus of digestive cell; L, nucleus of lime cell. Scale—40 μ.

proof for Heaysman's implication that this is the only or even major route: the method by which she traced the calcium, staining the tissues in galamine blue, gives no direct evidence of its passage through the epithelium of any part of the gut; this method is probably not sensitive enough to detect minute quantities of calcium. The work of van Weel (1950) shows, without doubt, that diffusion of iron saccharate from the lumina of the tubules into both types of cell of the digestive gland occurs in the African Giant Snail, *Achatina fulica*, and it is probable that calcium follows the same course.

If a slug be fixed $1\frac{1}{2}$ hours after commencing to feed, autoradiographs of the tissues show that some phosphorus has passed into the digestive cells of the gland, and some into the lime cells; in both it is dispersed in the cytoplasm. After 9 hours the concentration in the lime cells (fig. 1A, L) is greater than that in the digestive cells (D). Autoradiographs of animals which are fixed 48 hours after the commencement of feeding on P^{32} show the deposits still scattered through the digestive cell (fig. 1B, D), whereas in the lime cell (L) they are focused in large numbers around each spherule of calcium (C), sharply defining it on the film. Some of the tracer element in the lime cell may have

entered from the blood, for by this time it has circulated to all regions of the body, and is accumulating in specific tissues; the film indicates dense patches in the basal cytoplasm of some of the cells. Even after several meals of lettuce with P^{32} there is no heavy concentration in the digestive cells: from them there is a steady diffusion into the blood. Specimens of *Arion hortensis* which have been fed on contaminated lettuce for 7 days, and then for a fortnight on uncontaminated leaves, still show a random dispersal of the phosphorus through the cytoplasm of the digestive cells; less in the cytoplasm of the lime cells; but heavy concentrations in the calcium spherules. The phosphorus in the digestive cells would be concerned with immediate metabolic activities; in the lime cells it appears to be concentrated as a reserve. Corresponding experiments with *Helix aspersa* and *H. pomatia* give similar results.

The digestive gland is not the only site of uptake of food from the gut, though it offers a larger area than any other. A little of the phosphorus passes through the wall of the crop, but more through the wall of the intestine. The permeability of the wall of the intestine to iron has already been observed in *Helix pomatia* (Jordan and Lam, 1918; Hörstadius-Kjellström and Hörstadius, 1940) and *Achatina fulica* (van Weel, 1950), the iron diffusing slowly through the epithelium. Experiments with P^{32} show that this permeability is most rapid in a snail immediately after hibernation, when the higher metabolic rate of the animal is being restored. Vonk, Mighorst, and de Groot (1950), using heavy water as a tracer, found that at such a time the synthesis of glycogen in *H. pomatia* was surprisingly high. Specimens of *H. aspersa* which were fed at the first sign of activity after their winter torpor, and were fixed 5 hours later, showed a scanty flow of P^{32} through the epithelium of the oesophagus, and much larger amounts passing through the wall of the intestine, where the food may be retained longer, and still has a high proportion of undigested material. The deposits are not concentrated in any particular cells in this region of the gut, but appear haphazardly scattered in all the tissues and in the underlying haemocoel. After the intestine has emptied, an indication of phosphorus in its epithelial cells is sometimes evident. This may be correlated with the presence of phosphatase, which is described in this position and also in the wall of the oesophagus by Wagge (1948).

The Concentration of P^{32} in Certain Tissues

P^{32} passing into the blood will be taken up by the tissues and built into organic phosphorus compounds, labelling the molecules synthesized from it. Since the state of the tissues is not permanent, but is constantly in a state of breakdown balanced by synthesis, the isotope will make itself evident not only in the newly formed cells, but in tissues which are already established. It will indicate the rate of renewal of these tissues. Phosphorus is one of the most abundant mineral elements in the body of an animal, and is unique among the inorganic nutritional elements in that it is involved in the mechanism for the mobilization and storage of energy for synthesis; it is essential to every cell. Consequently there are many aspects of its metabolism which

might be studied with the tracer element. The present investigation is concerned only with some of the more unexpected concentrations of P^{32} in the tissues of the pulmonate, and, in some cases, suggests an explanation for these.

If autoradiographs of the body of a slug or snail which has been fixed (6 hours after a meal) be examined microscopically, it will be found that all the tissues have taken up at least a little of the phosphorus. The distribution within the body alters with time: an animal which is fixed 5 days after its first

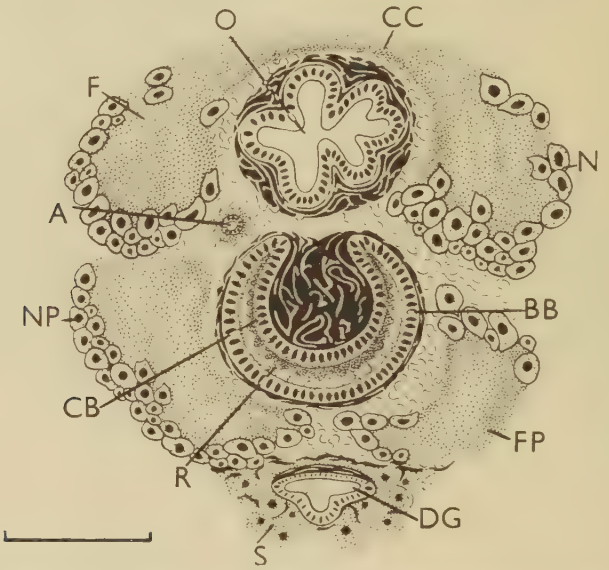

FIG. 2. Transverse section through the nerve ring and associated structures of *Agriolimax agrestis* to show the accumulations of P^{32}. Stippling represents the superimposed autoradiograph. Fixed neutral formaldehyde 5 days after commencing to feed on the tracer. A, anterior aorta; BB, basoblast; CB, cuspidoblast; CC, cerebral commissure; DG, duct of pedal mucous gland; F, nerve-fibres of cerebral ganglion; FP, nerve-fibres of pedal ganglion; N, nerve-cell of cerebral ganglion; NP, nerve-cell of pedal ganglion; O, oesophagus; R, radular tooth; S, secreting cells of pedal mucous gland. Scale—300 μ.

meal with P^{32} shows more selective accumulation, and radiations from the element are concentrated in the ring of ganglia and its accompanying nerves, the pedal mucous gland, the salivary glands, the odontophore, and certain cells of the mantle. The accumulation in the digestive gland will be neglected for the present.

In the nervous system the deposition occurs around each individual nerve-fibre and less around the nerve-cell. It is present in a mature animal 5 days after its first meal with P^{32}: autoradiographs show the centre of the ganglion, which is composed of very fine fibres (fig. 2, F), as a blackened area, the surrounding nerve-cells (N, NP) faintly indicated, with the boundary of each mapped out more distinctly by the radiations. Such activity, however, is only evident in frozen sections: in material fixed in alcohol there are fewer radiations, diffusely scattered over the whole area. It must therefore indicate a

compound which is soluble in alcohol: in such a position it is probable that there are phospholipines, which, as in other invertebrates, are associated with medullated nerves.

A lipoid sheath surrounds the giant nerve-fibres of the stellar nerves of *Loligo* (Bear, Schmitt, and Young, 1937), the giant fibres of the earthworm (Friedlaender, 1889), and the claw nerves of the lobster and crayfish (Bear and Schmitt, 1937). Chinn (1938) has shown that in the crustaceans the unipolar nerve-cells are also enclosed in a sheath containing a thin lipoid layer, and this is continuous with the axone sheath. The nervous system of *Limulus* and of the spider crab *Libinia* have a high concentration of lipoids—with much phospholipine—and these have been analysed by McColl and Rossiter (1950). They suggest that in these and other invertebrates lipoids are contained in a sheath that surrounds the axone and many of the cell-bodies. If a myelin sheath occurs in the pulmonates it is very thin, for it cannot be revealed by staining with osmium tetroxide. This, however, is not surprising since Young (1936) was unable to demonstrate the presence of the sheath in cephalopods in this way. In cephalopods it measures 1 per cent. of the diameter of the axone and in the much finer nerves of the gastropod it is improbable that it is relatively thicker. Pumphrey and Young (1938) suggest that in all molluscs the conduction rate of the nerve-fibres is proportional to their diameter, and not, as in other animals, to the thickness of the myelin sheath: the slow conduction in the gastropod, 0·5 m. sec. or less, is correlated with very fine fibres which are most probably as poorly medullated as the nerves of cephalopods.

In the wax moth, *Galleria melonella*, Lindsay and Craig (1942) note the presence of P^{32} in the nerve-cord in frozen and paraffin sections, but they do not mention whether the concentration in the two types of section is equal. They state that the presence of the isotope is of interest 'because of the low concentration in the brain of vertebrates and may be associated with the probable absence of myelin sheath'. However, since other arthropods possess a sheath it is probable that the insects do as well, though it may be of negligible thickness.

Radiophosphorus accumulates rapidly in the mucous glands of the pulmonate at a rate proportional to the activity of the glands. The pedal gland (fig. 2, DG, S), which opens between the propodium and mesopodium, has a heavy deposit 9 hours after a meal, and this is scattered in the cytoplasm surrounding the spherules of mucus, and is liberated with the secretion. As time increases a greater concentration is apparent; it is seen in autoradiographs of tissues which have been fixed either in absolute alcohol or neutral formalin. Since mucus is a glycoprotein containing carbohydrate not combined with phosphoric acid, such a rapid and continuous incorporation of phosphorus into the mucous cell—making the gland in autographs conspicuous against the surrounding muscle —seems, at first, unaccountable. However, the mucus of a snail (Prenant, 1924) and of a slug contains calcium which is said to be present as the carbonate. It is possible that in the presence of excess phosphate some calcium phosphate is formed. This occurs in the calcium stores of the body.

Robertson (1941) regards the calcium present in mucus as an excretion of excess calcium absorbed with the food. It is perhaps better to think of it as an essential part of the secretion. Divalent ions such as calcium and magnesium stabilize intercellular matrices and the surfaces and mucous coverings of cells and organisms. So the calcium in the mucus of the pedal gland would prevent the immediate dispersion of this secretion, which is poured out to make a pathway for the animal, and must remain tacky for a while to secure it a foothold. In *Procerodes* calcium for this purpose is provided by the sea-water (Weil and Pantin, 1931) and without it the mucus, as an aid to locomotion, is ineffective. The land pulmonate must provide its own calcium, and this may be stored as carbonate or phosphate. It is relevant to note that the slime of the terrestrial triclad, *Rhynchodemus*, also abounds in calcium (Percival, 1925; Eastham, 1933).

The pedal mucous gland comprises one of the largest accumulations of mucous cells in the body, and so is most obvious as an area of great activity from the isotope. Solitary glands, however, take up the phosphorus whether they be situated in the mantle or in the epithelium of the gut, or are associated with the reproductive system. Large quantities of mucus are continually produced by the salivary glands. The saliva also contains enzymes: amylase, xylanase, and saccharase (Jordan, 1913; Krijgsman, 1925), and in localized regions of the gland phosphatase is present (Wagge, 1948). Some of the P^{32} which enters the salivary tissue will therefore become involved in the various metabolic activities of the cells and with the mucus. The heaviest concentration of the phosphorus occurs in cells which are isolated from one another and give no positive stain for mucus. Their cytoplasm is vacuolated and the vacuoles contain spherules which are soluble in acid; it is in the cytoplasm that the phosphorus is heavily deposited.

The concentration of the isotope in the odontophore is associated with the high metabolic rate of the muscles, and some deposits can also be traces in the radular teeth (figs. 2 and 3B, R). The radula of a mollusc is produced by secretion from two sets of cells, the basoblasts (BB) and the cuspidoblasts (CB). The teeth are formed in closed follicles which are lined by these cells, and no cell is concerned with the production of more than one tooth or part of a tooth; a number of basoblasts fuse together to form its base, and cuspidoblasts secrete the cutting edge (Pruvot-Fol, 1926). The activity of the cells is displayed by radiophosphorus. The isotope, circulating in the blood sinuses of the radular sac, is rapidly incorporated into their cytoplasm; autoradiographs of the odontophore of *Agriolimax* show that this may occur within 10 hours of feeding. Some hours later it can be seen from other specimens that the phosphorus passes into the 'chitin' which is being secreted, and this gives an indication of the relative speed at which the two types of cell are producing a single tooth. At the very tip of the radular sac, where new teeth are beginning to form, the basoblasts are the active cells, and the cuspidoblasts may not be secreting, or, if they are, their rate of secretion is very slow. In passing forwards along the sac it can be seen that the basal cells are com-

pleting their task and are gradually becoming effete, and, as they do so, the cuspidoblasts accelerate their production of 'chitin'; they alone add to the more anterior teeth (fig. 3B, CB). Experiments with *Helix* give similar results. Once a tooth is liberated from its cuspidoblasts there appears to be no addition to its substance. The tracer can, however, be seen on the used cusps, adsorbed to their surfaces. The reason for the incorporation of the phosphorus into the radular teeth is not known.

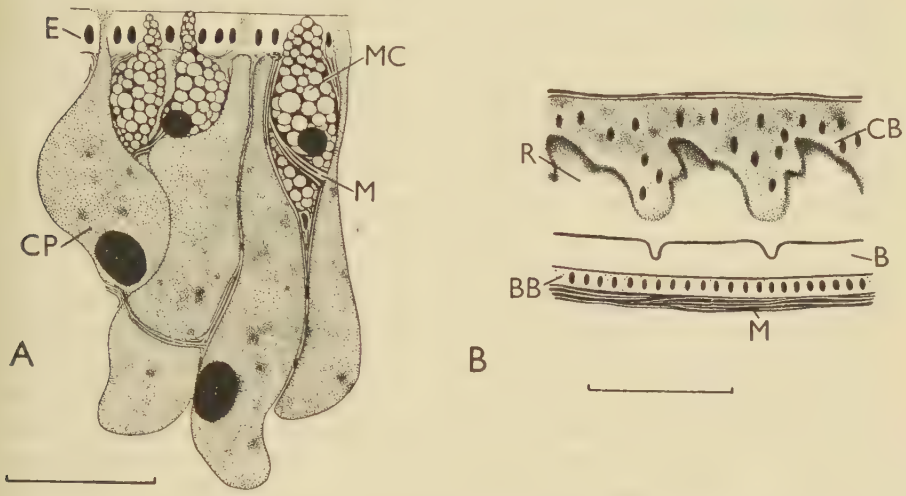

FIG. 3. A, Part of transverse section through the lower region of the mantle edge of *Helix pomatia* to show concentrations of P^{32}. Stippling represents the superimposed autoradiograph. Fixed absolute alcohol 12 days after commencing to feed on the tracer. B, Part of transverse section through the developing teeth of the radula of *Arion hortensis* to show the distribution of P^{32}. Stippling represents the superimposed autoradiograph. Fixed neutral formaldehyde 10 days after commencing to feed on the tracer. B, basal plate of radular tooth; BB, basoblast; CB, cuspidoblast; CP, calcium gland; E, epithelium of mantle; M, muscle; MC, mucous cell; R, radular tooth. Scale—A, 80 μ; B, 70 μ.

In *Helix aspersa* and *H. pomatia* the cells of the mantle which accumulate the P^{32} are in the edge of the skirt. In the larger of the two snails, *H. pomatia*, it can easily be seen that this edge has a deep groove along its length, and its upper part, containing the groove, is muscular and pigmented, whilst the lower part is tumid and has many subepithelial glands. In some of these glands the phosphorus is present in large quantities (fig. 3A, CP), and in others—typical mucous cells—there is a smaller amount (MC). Prenant (1924) describes this glandular area as being made up of calcareous and mucous cells and figures the distribution of these. Heaysman (1951) disagrees, and believes that the calcium is not in cells, but in blood-spaces; though her photograph of a section through the mantle edge shows calcium deposits identical in size, shape, and position with the cells which take up large quantities of radiophosphorus, and agree with the calcium cells of Prenant (1924). She suggests that calcium may be stored here as a carbonate or a phosphate, and that these

supplies are used in the growth of the shell. Thus it would seem that P^{32} is taken up from the blood by the calcium stores in the mantle edge, and may be combined with the calcium to form calcium phosphate. In *H. aspersa* the phosphorus accumulates in similar cells, and specimens of both species of snail, which have been fed with the tracer and fixed 3 weeks later, still show a heavy accumulation in this position.

The amount of phosphate in the shell of molluscs may be very slight. Pelseneer (1906) considers that they all contain 1–2 per cent., though this may be disputed. Heaysman (1951) found a trace in the shell of *Helix pomatia*, and none in the shells of *Nucella lapillus* and *Paludina vivipara*. Bevelander and Benzer (1948) describe granules of calcium phosphate in the mantle epithelium of five lamellibranchs—*Atina rigida*, *Pedalion alatum*, *Codokia*, *Pinctada radiata*, and *Venus mercenaria*—and say that at calcification these granules are deposited in the conchiolin layer of the shell and begin to undergo a typical crystal growth. These authors, however, state that the final calcified product in the shell is calcium carbonate (calcite); they are unable to explain the change from a phosphate to a carbonate.

In the slug the shell is internal: it is composed of scattered particles in *Arion hortensis*, and forms a thin cap in *Agriolimax agrestis*. Some of the cells of the mantle which secrete it take up P^{32}, and this, especially in a rapidly growing slug, is seen in the shell 2 days after it has taken a meal which contains the isotope. Other cells of the mantle which are exposed on the surface of the body, dorsal to the shell, and are scattered in the epithelium with the mucous cells, also take the tracer element. They contain calcium (Barr, 1928) with which the phosphorus combines.

Mention may be made of the accumulation of the phosphorus by nuclei. Autographs of young animals, as compared with those of adults, show a much greater concentration of activity in nuclei. Most of the phosphorus would be present as desoxyribonucleic acid phosphorus and the turnover of this compound in cells undergoing regular mitosis, as in a growing animal, must be appreciable (Marshak, 1941).

Storage of P^{32} in the Lime Cells of the Digestive Gland and its Transport by Amoebocytes

Calcium is an important mineral element in all animal tissue and in the majority of molluscs its significance is emphasized by their possession of a thick calcareous shell. The study of calcium metabolism, especially in land snails since they are ubiquitous and vigorous, has, therefore, attracted many workers. These have established the fact that in both *Helix aspersa* and *H. pomatia* the lime cell of the digestive gland is the storage place for this element. Within the cells the calcium is deposited inside minute spherules of protein. Krijgsman (1928), Sioli (1935), and Manigault (1939) hold that the store in *H. pomatia* consists of calcium phosphate crystals, and suggest that the phosphate may act as a buffer on the intestinal contents. Some of the calcium is used in shell growth, and Sioli (1935) and Manigault (1939) agree

that during the regeneration of a broken shell there is a marked fall in the calcium content of the digestive gland. But, according to Sioli (1935), this loss of calcium from the gland is not accompanied by any loss of phosphate. More recently Heaysman (1951), in studying the calcium deposits of *H. pomatia*, states that the crystalline form in the digestive gland is aragonite, the only calcium phosphate being, apparently, in the mantle edge. Wagge (1948), too, finds that in *H. aspersa* the calcium in the digestive gland is combined with carbonate. The carbonate would almost certainly be obtained by the retention of metabolic carbon dioxide; such is the source in the embryonic shells of gastropods (Baldwin, 1935).

There is thus controversy as to the chemical form of the calcium store in the liver of *Helix*. With this in mind Wagge (1948) fed specimens of *H. aspersa* on a diet rich in calcium phosphate to see whether the rate of absorption of the calcium was more rapid than from a similar diet with calcium carbonate. Although, as she admits, no conclusive results can be drawn from some of the experiments, since they involve only single animals, there is evidence to suggest that calcium carbonate is absorbed more readily than monobasic calcium phosphate.

Radioactive tracers provide a new approach to the study of the lime cells in the digestive gland. Feeding experiments show that in *Helix aspersa*, *H. pomatia*, *Arion hortensis*, and *Agriolimax agrestis* the phosphorus is taken up directly into the cytoplasm of these cells from the lumina of the tubules of the gland; and it appears to enter the cell from the haemocoel, which has received it either from the digestive cells or from the epithelial cells of the gut. The lime cells are recognized as the site for the accumulation of calcium reserves: they also gather the excess phosphorus which is taken into the body. Autoradiographs show that in no other type of cell is there such a concentrated store of the tracer. Presumably it combines with the calcium as a calcium phosphate reserve: this has already been recognized in *Helix pomatia* by some workers; it also occurs in *H. aspersa*. A few specimens of *H. aspersa* were given a dose of P^{32} equivalent to 4μ C. per gram of tissue, this being consumed in four days, and were then fed on uncontaminated lettuce for a period varying from 1 to 3 weeks. Autoradiographs showed that even after 3 weeks the phosphorus is still most abundant in the lime cells, concentrated at each protein spherule. Yet only calcium carbonate reserves have been found by chemical tests on the liver of this species. It would appear that in the pulmonate calcium may be present in the digestive gland as a carbonate (Wagge, 1948; Heaysman, 1951); or calcium and phosphorus, the two most abundant mineral elements in the body, and each with a number of important roles, may be stored together as calcium phosphate. Although in these experiments the activity of the tracer in the lime cells appears high the amount of phosphorus is actually very small. However, it shows that the animals are able to retain a reserve of phosphorus. It is probable that the maximum storage capacity of phosphorus is much greater than could be tested with the radioactive isotope which would injure the tissues.

Wagge (1948) has made a careful study of the function of amoebocytes in the regeneration of the shell of *Helix aspersa*, and has shown that they transport calcium reserves from the liver to the site of regeneration; this has been repeated by Heaysman (1951) for *H. pomatia*. In order to find out whether phosphorus is also carried by these wandering cells to the region of repair, some experiments were carried out on the regeneration of the shell of snails which had been fed with P^{32}. Small pieces of shell were removed from specimens of *H. aspersa* and *H. pomatia*, which were active and feeding well, and similar results were obtained for both. Regeneration of the injured part is rapid, and after 1 day the new pieces of shell may be too thick for detailed microscopic examination and for the film stripping. Within this time a thin regenerating part may be removed from the shell, spread on a clean slide, and fixed for 5 minutes in absolute alcohol; after dehydration the film stripping may be floated on to it. It is necessary to decalcify the thicker pieces of shell, and for this acetic or formic acid may be used; the inorganic acids, nitric and hydrochloric, remove the radioactive material during decalcification (Siffert, 1948), giving unsatisfactory autographs. The experiments show that amoebocytes of two sizes are active during regeneration. Clusters of them can be identified, each carrying the isotope: this may be associated with alkaline phosphatase in the smaller amoebocytes, but the larger ones have not been found to contain the enzyme (Wagge, 1948) and the phosphorus may be combined with the calcium which they carry. Phosphorus is also present in the regenerating parts of the shell, indicating, perhaps, some deposition of calcium phosphate which will be converted into calcium carbonate later (Bevelander and Benzer, 1948).

According to Wagge (1948) the amoebocytes of both sizes bring the calcium from the stores in the digestive gland to the surface of the body, and presumably the phosphorus is collected simultaneously from the same site. Sections of two young *Helix aspersa*, in which the shells were regenerating, showed groups of amoebocytes, containing the tracer element, both beneath the tubules of the digestive gland and near the locus of repair.

In the slug, also, the isotope is found in amoebocytes (fig. 1B, AM). A specimen of *Arion hortensis* which had been fed on contaminated lettuce for 2 days had amoebocytes beneath the tubules of the digestive gland, and some in contact with the lime cells, the cytoplasm and the nuclei of both showing a high activity; it may be that they obtained the radiophosphorus from the lime cells. Similar wandering cells were found in other regions of the body, though the role they play in the metabolic activities of the slug has not been investigated.

The slug and other gastropods have appreciable calcium deposits around the blood-vessels. After feeding a slug on P^{32} activity is seen in this storage tissue (fig. 2, A). It presumably denotes the presence of calcium phosphate, the phosphate having been taken from the blood. In one specimen of *Arion hortensis* groups of amoebocytes containing the tracer were seen against the anterior aorta; they may be either augmenting or depleting the stores.

Feeding Experiments with I^{131}

If a slug or snail be fed with lettuce on to which I^{131} has been dried, autoradiographs of the tissues show that the distribution of the iodine is in many respects different from that of phosphorus. Animals which eat approximately 4μ C. per gram of body weight in 2 days, and are then fixed, show iodine in greatest abundance in the absorbing cells of the digestive gland, where it is scattered throughout the cytoplasm. In contrast to the results obtained with P^{32} the lime cells have many fewer deposits than the digestive cells, and there is no tendency for them to concentrate around the calcium spherules. Even though the dose be nearly doubled, and be taken in by the pulmonate over a period of 7 days, the lime cells show no concentrated accumulation. Some iodine, but little, passes into the epithelium of the intestine: in fact, its passage from the lumen of the gut is by way of the same cells as have been described in the uptake of radioactive phosphorus, though the relative proportions appearing in the cells are different.

From the absorbing cells phosphorus passes rapidly into the haemocoel, whence it is distributed and accumulates in specific loci. Only a very small amount of iodine appears to leave these cells by this route: in no tissue of the body is it stored. Autoradiographs of individuals which have been fed on I^{131} over a period of 2 days or longer show a scanty dispersal throughout the body, with the exception of the digestive gland. Most of the material thus dispersed must have passed from the gland—some, perhaps, coming from the epithelium of the gut—and this is, presumably, associated with ionic exchange (Brooks, 1939). What remains in the digestive gland is eventually extruded to the lumen of the gland, and may be traced in the faeces of animals which have been fed on uncontaminated lettuce after a dose of I^{131}. Van Weel (1950) suggests that it is by this same route that iron saccharate, absorbed by the digestive cells of *Achatina*, and also valueless to the animal, is then freed from them. I am uncertain whether the lime cells lose their iodine in a similar way, though it is probable that they do.

The feeding experiments with P^{32} and I^{131} would thus suggest that the epithelium of the digestive gland of pulmonates may take up from the food any substances, whether they be of metabolic value to the animal or not. But the passage of these into other tissues of the body is governed by the demand for them. In *Drosophila* (Wheeler, 1947), however, I^{131}, although still useless to the animal, is taken in with the food and passed freely into the body to accumulate in the exoskeleton, which thus forms a depository for waste matter, and is cast away at the next moult. The pulmonate has no such provision for storing, away from the living tissues, relatively large quantities of valueless or harmful substances, and the passage of these into the body from the digestive gland appears to be restricted. The iodine which is dispersed accumulates slowly in the kidney, whence it is excreted. In the slug, however, some of the deposits from the kidney pass into the closed-off portion of the mantle cavity, which lies above it and contains the shell, and in some

specimens of *Arion hortensis* the I^{131} may be centred around the scattered shell particles.

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Studies upon the Gram Reaction of the Basiphil Cells of the Anterior Pituitary

Part I. Some Preliminary Observations upon the Basiphil Cell of the Human Pituitary

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With two plates (figs 1 and 2)

SUMMARY

The basiphil cell granules of the human anterior pituitary react positively with Gram's stain, but no specific differentiation of these granules occurs when the iodine treatment is omitted. The granules of the acidophil cells are Gram-negative.

Various workers have suggested a connexion between the Gram reaction of various micro-organisms and the presence of ribonucleoprotein, and the pituitary cells were investigated from this point of view.

Pyronin methyl green staining demonstrates what is probably ribonucleoprotein material in the cytoplasm of both chromophobes and chromophils, but not in the granules of either kind of chromophil. As with micro-organisms, the Gram reaction is destroyed by treating the sections with hot oxygenated bile salt, which has a detergent action upon the ribonucleic acids, but, unlike micro-organisms, the reaction remains unaffected by digestion with a buffered solution of crystalline ribonuclease, although this treatment destroys the nucleic acid, as demonstrated by the loss of pyronin basiphilia in the cytoplasm.

Bile salt also diminishes the strong positive reaction of the basiphil cell granules to the periodic acid Schiff (P.A.S.) test—a reaction which probably indicates the presence of intragranular mucoprotein.

It seems unlikely that the Gram-positive reaction of the basiphil cell granules of the human anterior pituitary is due simply to the presence of ribonucleoprotein.

We have employed the term ribonucleic acid, bearing in mind that this is a generic term and not the name of a single molecular species (cf. Davidson, 1950).

INTRODUCTION

IN a short communication (Foster and Wilson, 1951) we recently reported, in brief, the results of a preliminary investigation into the Gram-positive properties of the cells of the human anterior pituitary. The present paper is a full account of this work.

There is a very extensive literature upon the Gram reaction in relation to bacteria and other micro-organisms. It falls, with a considerable amount of overlap, into two categories. The first deals primarily with the chemistry of the Gram reaction as such, and the more important opinions and conclusions of the numerous investigators concerned are critically discussed in

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recent papers by Bartholomew and his colleagues (Bartholomew and Mittwer, 1950; Mittwer, Bartholomew, and Kallman, 1950). The second group includes researches whose main objects have been to discover the chemical nature and location of the cellular material which reacts positively in the Gram technique. This aspect of the problem is admirably summarized in a recent article by Mitchell and Moyle (1950).

Little, however, seems to be known about the Gram reaction as it affects the tissues of higher organisms, and the writers' attention was drawn to this problem by a brief statement of Pearse (1949) that the basiphil cells of the human anterior pituitary were Gram-positive. In view of our interest in the ribonucleic acid distribution in pituitary cells and the fact that the experiments of Stacey and his colleagues (Henry and Stacey, 1943, 1946; Stacey, 1947) seemed to indicate that this substance is intimately concerned in the mechanism of the Gram reaction in micro-organisms, we decided to investigate the problem with particular reference to the human pituitary.

The methods described in this paper, which are those we initially used, are largely based upon those of Henry and Stacey (1943, 1946). One difference should, however, be pointed out at the outset. It is that whereas most workers studying the Gram reaction in bacteria and similar organisms have fixed their material by heat treatment, our pituitary specimens were fixed in 10 per cent. formaldehyde-saline, paraffin embedded and sectioned in the usual manner. We hope to study the effect of other methods of fixation, including heat, upon the Gram reaction during the course of the further work which is now in progress.

Materials and Methods

Human pituitaries were used and were fixed as soon as possible post mortem in 10 per cent. formaldehyde-saline. Paraffin sections from 7 to 10 μ in thickness were used for study, and several sets of these were arranged in serial order. The further procedures may be classified as follows:

(*a*) *Bile extraction*

The method used followed very closely that described by Henry and Stacey (1946). The detergent action of a 2 per cent. aqueous solution of sodium tauroglycocholate was used to free the ribonucleic acids from their protein attachment. There is no reason to believe, however, that this substance has a specific action in this respect, although it may be that other materials are removed in relatively small amounts. The extraction was carried out over a period of at least 24 hours at 60° C. and the solution was kept continuously oxygenated. Continuous oxygenation is very important, and the incomplete removal of Gram-staining material occasionally encountered, even after prolonged extraction, could always be attributed to imperfect oxygenation.

(*b*) *Ribonuclease digestion*

A solution of crystalline ribonuclease was used, buffered to pH 6·8 in a citrate-phosphate buffer. Sections were incubated at 37° C. for 5 hours, and

sometimes longer, in a solution containing 0·25 mgm. of enzyme per ml. of buffer. Accurate buffering was found to be necessary to ensure the effective destruction of nucleic acid, as measured by the disappearance of the cytoplasmic pyronin basiphilia. The activity of the enzyme was also checked by incubating formalin-fixed sections of human pancreas at the same time. Control specimens were incubated in buffer solution only.

(*c*) *The periodic acid Schiff* (*P.A.S.*) *technique*

The procedure followed was that of Pearse (1949). Although the specificity of this reaction for mucoproteins and allied substances is clearly open to question (Lillie, 1950), there is no doubt that it is a very convenient method for the demonstration of the pituitary basiphil cells, and serial sections show that there is a very close correspondence between the distribution and characteristics of P.A.S.-positive cells and those which would be designated as basiphils by the standard staining techniques. Moreover, there is a reasonable probability that the positive reaction of the basiphil cell granules is indicative of the presence of mucoprotein in their composition. Acidophil cells were demonstrated by counterstaining with a 0·5 aqueous solution of orange G.

(*d*) *'Replating' with magnesium ribonucleate*

These experiments were undertaken to see whether it was possible to 'replate' the basiphil cells whose ribonucleic acid and positive Gram staining had been destroyed by bile salt extraction. Two methods were used, and, in both instances, the sections were first thoroughly washed in running tap-water on removal from the hot bile-salt solution.

In the first method, the slides were placed in a reducing solution of 1 per cent. formaldehyde for several hours or overnight, and were then transferred for at least 3 hours to a filtered solution of 0·5 gm. $MgCl_2$ and 0·5 gm. sodium ribonucleate dissolved in 400 ml. of distilled water. Bile-extracted sections of formalin-fixed human pancreas were taken through at the same time: pancreas was chosen because of its high content of ribonucleoprotein present in the form of 'chromidial' substance. In both cases, bile-extracted but otherwise untreated sections were used as controls as a check upon the effectiveness of the extraction.

In the second method, magnesium ribonucleate was prepared in the way described by Bartholomew and Umbreit (1944). 0·5 gm. of sodium ribonucleate was thoroughly shaken up in 500 ml. of 1 per cent. $MgCl_2$ and then filtered. To the filtrate N/10 HCl was added drop by drop until maximum turbidity was obtained. The suspension of magnesium ribonucleate so formed was allowed to settle out for about an hour and was then filtered through a Buchner funnel. The precipitate was scraped off the filter-paper, and as much as possible was redissolved in 200 ml. of 0·1 per cent. formaldehyde and then brought to neutrality by the addition of a small quantity of magnesium carbonate. From this point the procedure was similar to that previously described.

In both cases the degree of 'replating' was estimated by comparison with bile-extracted control sections, which were stained in a pyronin methyl green mixture at the same time.

(*e*) *Staining methods*

For the routine identification of cell types, Mann's methyl blue-eosin and Mallory's acid fuchsin, orange G aniline blue mixtures were used.

Basiphilia due to ribonucleoprotein was demonstrated by using a pyronin methyl green mixture made up according to the formula of Baker (1942). With different samples of pyronin it sometimes became necessary to alter the proportions of the two dyes. This was done empirically, with the formalin-fixed pancreas as a convenient test material.

As was mentioned in a previous paper (1951), several variants of the Gram technique were tried, but the one which gave the best results was as follows:

1. Bring section down to distilled water and flood with a 1 per cent. aqueous solution of crystal violet for 3 minutes.
2. Rinse in tap-water.
3. Shake off surplus water and cover with Lugol's iodine for 3 minutes.
4. Blot well, and differentiate in clove oil.
5. Rinse in xylene and mount in Canada balsam.

(Note. In stages 1 and 3, periods of 2 minutes gave equally good results.)

According to bacteriological procedure (Bartholomew *et al.*, 1950), the Gram technique must, by definition, include a counterstain which should neither mask nor displace the crystal violet iodine complex. It was found that the Gram staining of the basiphil granules was resistant to a 0·5 per cent. solution of safranin O. in water, although, as a routine procedure, the counterstain was omitted.

When the iodine treatment was omitted it was invariably found that the dye was rapidly removed by the clove oil, with no specific differentiation of the basiphil granules.

Results

The identity of the Gram-positive cells with basiphils

The first point which had to be established in this work was the nature of the pituitary cells, the granules of which were reacting positively to Gram's stain. Examination of the Gram-positive cells showed, quite unequivocally, that the dye complex was located only in cytoplasmic granules.

Of the chromophils, the Gram-positive ones resembled the basiphil type in all important respects. The cells were somewhat larger and more irregular in shape than acidophils, whereas the granules were smaller. The cytoplasm also showed single or small groups of largish vacuoles, which seem to characterize the pituitary basiphils of elderly human subjects.

Further evidence in support of this identity was obtained by the use of serial sections. One section of a series was stained by Gram's stain, and the

next by the periodic acid Schiff technique (fig. 2A) (which appears to be specific for basiphils (Pearse, 1949, but see also McManus, 1948)), or occasionally with Mann's stain. Microscopical examination always showed a strikingly close correspondence between the distribution of the P.A.S.- and Gram-positive cells. Further, the similarity in cytological appearance between the cells demonstrated by these two methods was very marked (see figs. 1B and 2C).

Fig. 2D shows an example of the curious vesicles with a P.A.S.-positive cortex which were frequently encountered in the basiphil cells of all the pituitaries we have studied. They are particularly evident in cells previously subjected to bile extraction. It is possible that the unstained central area is identical with the vacuoles mentioned earlier; the cortex can be quite deeply coloured with sudan black.

The collagenous connective tissue was found to be Gram-positive to some extent and was always P.A.S.-positive.

The distribution of ribonucleoprotein

On account of the views of Stacey and his colleagues with regard to the importance of ribonucleic acid in the Gram staining of micro-organisms, a considerable amount of time was devoted to this aspect of the problem. The presence of ribonucleic acid in the cells of the pars distalis of the human pituitary has already been described (Desclin, 1940; Dempsey and Wislocki, 1945). The latter workers reported a methylene blue basiphilia of the basiphil cells which could be destroyed by digestion with crystalline ribonuclease.

Using pyronin and methyl green we found that many of the pituitary cells contained strongly pyroninophil cytoplasmic masses of variable size and irregular shape. The granules, however, of both types of chromophil cell showed no characteristic basiphilia; they stained only an indifferent pink colour. Incubation with ribonuclease readily removed the cytoplasmic basiphilia, and the cytoplasm then stained a uniform rather faint colour, with the acidophil granules appearing somewhat darker. The same preparation of enzyme also quickly destroyed the cytoplasmic basiphilia of formaldehyde-fixed pancreas under the same conditions. In both instances, incubation in buffer solution alone was without effect. It was noted that the basiphilia was by no means restricted to a particular kind of cell, but was observed both in the chromophobes and the two kinds of chromophil. In some specimens a large proportion of the total basiphilic material appeared to be associated with the chromophobe cells. In order to test whether there was any marked correlation between the distribution of cells rich in ribonucleoprotein and Gram-positive cells, the following method was employed. Sections were first of all stained with pyronin and methyl green and a suitable small area of cells was sketched with the camera lucida, cells showing a distinctive basiphilia being suitably marked. The dyes were then removed with weak acid alcohol and the sections treated by Gram's method. Observation of the same areas once more showed that cells showing a strong basiphilia were very

commonly Gram-negative (e.g. chromophobes) and, conversely, cells with little or no basiphilia were frequently Gram-positive. No evidence could thus be found for any correlation between the distribution of nucleoprotein demonstrable by pyronin basiphilia and the Gram-positive reaction of basiphil cells. It must be remembered, however, that the technique used does not necessarily demonstrate *all* the ribonucleoprotein present in the cytoplasm or which might conceivably be present in the granules. (See Discussion.)

The effect of extraction with hot oxygenated bile salt upon the pyronin basiphilia, Gram staining, and periodic acid Schiff (P.A.S.) reaction of the basiphil cells

Hot bile salt readily removed the pyronin basiphilia from both the pituitary and the pancreatic sections taken through at the same time as a check on the extraction progress (fig. 1, E and F). There was also a destruction of the Gram reaction in the basiphils (fig. 1, A and D).

It was observed, however, that an extraction long enough to destroy the pyronin basiphilia was not necessarily sufficient to effect the complete removal of the Gram-stained material. In some specimens, extraction for at least 48 hours was necessary, even in conditions where there was no reason to suspect that the oxygenation was inadequate. The removal of the Gram-positive material from the basiphil granules thus appears to be a relatively prolonged operation. Examination of partially extracted sections also showed that the process does not take place uniformly, all gradations being observed between strongly positive and completely negative granules.

The application of the P.A.S. test to bile-extracted sections showed some diminution in the intensity of the reaction as compared with untreated controls (fig. 2D). Even, however, in sections subjected to the maximum degree of extraction used in these experiments (between two and three days), nothing approaching the total destruction of the P.A.S.-positive reaction was ever observed.

The effect of 'replating' bile-extracted sections with magnesium ribonucleate

It may be stated that, so far, attempts to replate magnesium ribonucleate on to bile-extracted sections have been unsuccessful both in pituitary and

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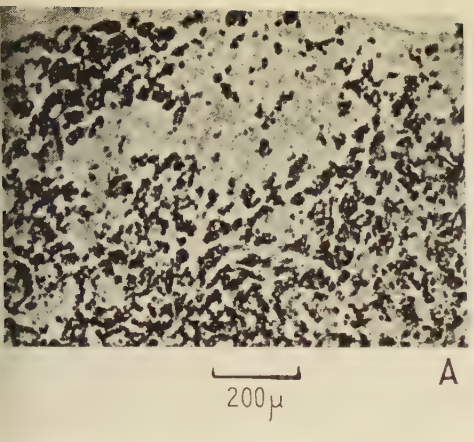
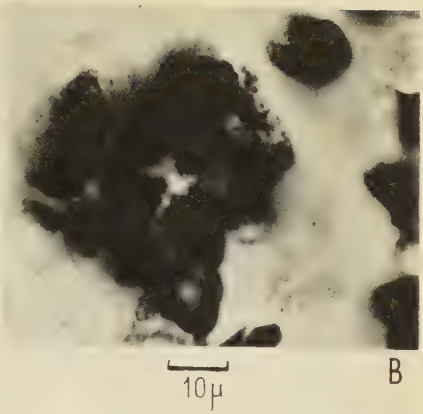
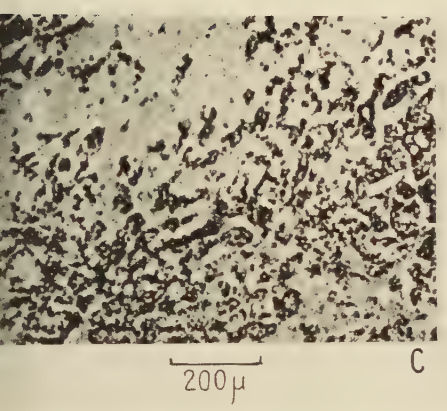
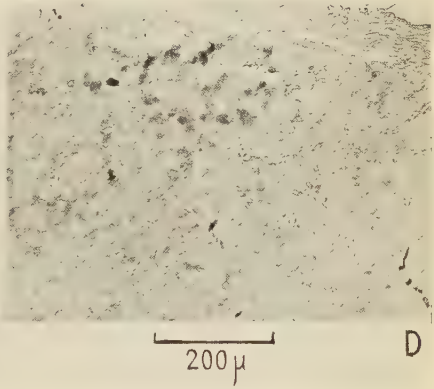
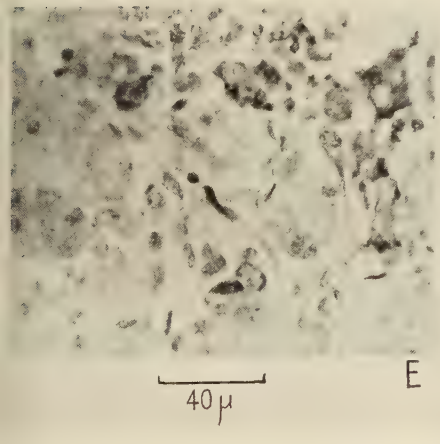
FIG. 1. All photographs in figs. 1 and 2 are of 10μ sections of human pituitary fixed in 10 per cent. formaldehyde-saline. A. Section treated by Gram's technique to show distribution of basiphil cells. B. High-power field of section prepared in similar manner to A. Some of the individual heavily stained granules can be seen at the periphery of some of the basiphil cells. C. Section treated with crystalline ribonuclease (0·25 mgm. per ml.) at 37° C. for 4 hours and subsequently stained with Gram's stain. The basiphil cells are still strongly Gram-positive. D. Section incubated with hot oxygenated bile salt for 24 hours. Almost complete destruction of Gram reaction (compare A). E. Section stained with pyronin and methyl green and showing dark areas of cytoplasmic basiphilia (particularly in upper right-hand part of the field). F. Neighbouring section to that shown in E. This was stained with pyronin and methyl green after extraction with hot oxygenated bile salt. Note the disappearance of the cytoplasmic basiphilia.











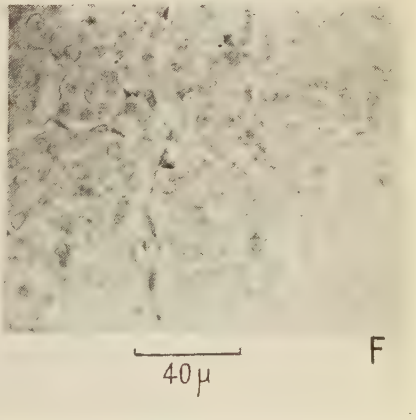


FIG. 1

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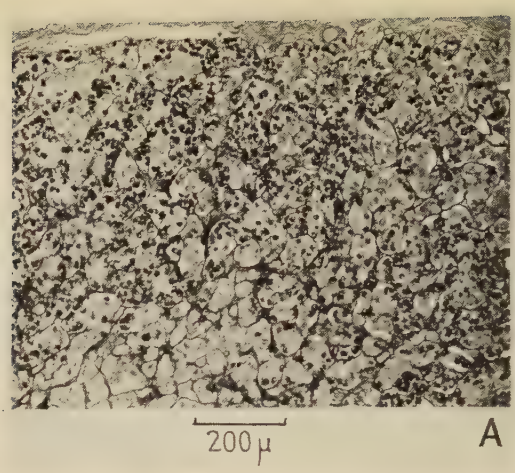

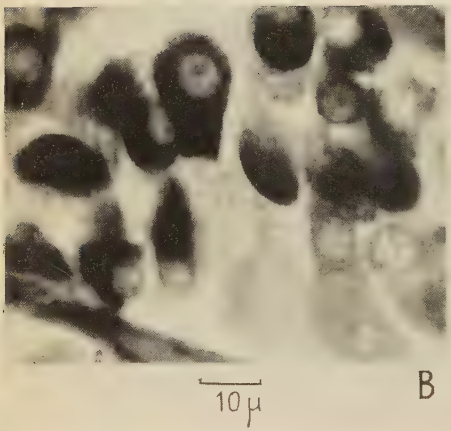

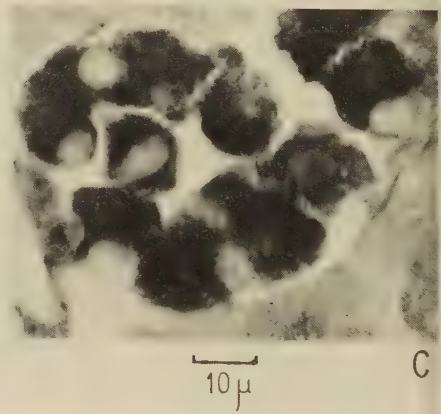

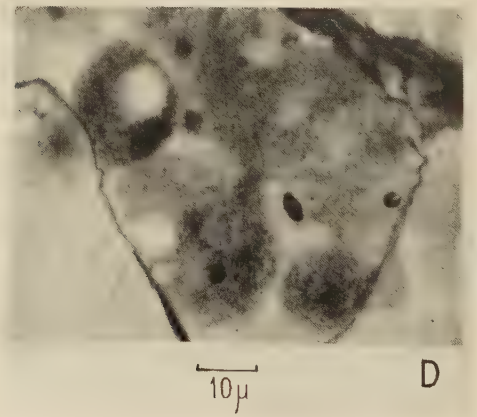

FIG. 2

C. L. FOSTER and R. R. WILSON

pancreas material. In the first set of experiments where an unbuffered mixture of sodium ribonucleate and magnesium chloride was used, there was undoubtedly an increase in pyronin-uptake as compared with the extracted but otherwise untreated control sections of both pituitary and pancreas. The staining, however, was completely lacking in specificity, since it involved the whole of the cytoplasm of the cells and the connective tissue as well; there was, moreover, no suggestion of the conspicuous reddish-purple tint which is normally associated with the staining of ribonucleoprotein. Furthermore, such sections were Gram-negative, as were their controls.

The results of the second set of experiments, with a solution of magnesium ribonucleate prepared in the manner already described, were even more negative, in that there was a very little generalized uptake of pyronin and once more a complete absence of Gram staining.

The effect of incubation with ribonuclease

As was mentioned earlier, the buffered solution of the enzyme was very effective in rapidly removing the pyronin basiphilia both from pituitary sections and the pancreas material used as a check upon the activity of the enzyme. It was found, however, that enzyme digestion was without effect upon the Gram staining of the sections, even after more than 5 hours' incubation at 37° C. (fig. 1C), whereas the pyronin basiphilia was completely destroyed after 2 hours or less. Since these findings after Gram's stain were not in accord with those of Pearse (personal communication, 1951), the experiments were repeated several times with different blocks of material, but the results obtained only confirmed our original findings. The enzyme was also without effect upon the P.A.S. reaction (see fig. 2, B and C).

Discussion

It would be helpful, before discussing them, to list the principal facts which have emerged from our experiments. They are:

1. The Gram-positive cells are the basiphils and it is their granules which react positively with the dye. Acidophil and chromophobe cells are Gram-negative. No specific differentiation of basiphils occurs when the iodine treatment is omitted, which suggests that the reaction is not just a simple staining of acidic granules by a basic dye.

2. Ribonucleoprotein is present in a proportion of *all* cell types, as is shown by the combined use of pyronin methyl green and ribonuclease digestion. This material is demonstrable in the ground cytoplasm but not in

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Fig. 2. A. Section treated by the periodic acid Schiff technique to show the distribution of the basiphil cells. B. High-power view of a section subjected to the P.A.S. technique after incubation with ribonuclease. The basiphil cells are still strongly reacting. C. High-power view of a section treated by the P.A.S. technique, for comparison with B. D. High-power view of a group of basiphils treated by the P.A.S. technique after bile-extraction for 48 hours. There is some weakening of the reaction (compare C) but the cytoplasmic vesicles with a P.A.S.-positive cortex are clearly seen.

the chromophil granules. It clearly follows also that there can be little correlation between the distribution of the ribonucleoprotein and Gram-positive cells.

3. Extraction with hot oxygenated bile salt destroys the pyronin basiphilia of all the cells as well as the Gram reaction of the basiphil cell granules. It also has the effect of diminishing the intensity of the P.A.S. reaction.

4. Digestion with ribonuclease has no appreciable effect upon the Gram reaction, although it destroys the ribonucleic acid.

If, as seems probable, the Gram reaction in micro-organisms is a reaction with a substance or complex of substances located in the cell membrane and surface cytoplasm (although this has recently been called in question (Hoffman, 1951)), there is no *a priori* reason why various intracellular granules of animals should not react in a similar manner. In other words, it is conceivable that some small granules might, from a physico-chemical point of view, be regarded as behaving like certain kinds of micro-organisms, in so far as the Gram reaction is concerned. It must be remembered, however, that bacteriological workers in this field have very largely used heat as their method of fixation, whereas we have used 10 per cent. formaldehyde-saline. The hypothesis just put forward would depend, of course, upon the not unreasonable assumption that intracellular droplets or granules possess an organized surface layer, comparable in some respects to the plasma-membrane of cells themselves. Even if this is true, however, the fact that both certain micro-organisms and basiphil cell granules are Gram-positive does not necessarily mean that reacting substances of a similar kind are located at their respective surfaces, the reason being that no cytochemical specificity can yet be claimed for the Gram reaction.

Nevertheless, we think it legitimate at the outset to admit the possibility that there may, in fact, be some underlying chemical similarity between all Gram-positive structures, until the contrary is established by experiment.

If our results are compared with those of Henry and Stacey (1943, 1946) and Bartholomew and Umbreit (1944), who have claimed that the Gram reaction is intimately connected with the presence of ribonucleoprotein at the bacterial cell surface, then they are in agreement in that the detergent action of hot oxygenated bile salt removes ribonucleic acid and destroys the Gram reaction in both instances. In the pituitary, however, there is no evidence for the presence of nucleic acids within the basiphil granules themselves; what is demonstrably removable by enzyme digestion is located in the cytoplasm.

Unlike Henry and Stacey, we were not successful in 'replating' the cells with magnesium ribonucleate, but this may have been due to a failure to achieve the correct conditions. More important than this, however, is the fact that digestion with ribonuclease was without effect upon the positive Gram reaction of the granules, although it has been claimed that this enzyme destroys it in various micro-organisms (Henry and Stacey, 1943, 1946; Bartholomew and Umbreit, 1944). It is, of course, still possible that ribo-

nucleoprotein is actually present in the granules, but that it is present in such a manner that it cannot be demonstrated by the pyronin methyl green technique or attacked by ribonuclease.

That ribonucleic acid is the essential factor in determining the Gram reaction of pituitary basiphil granules appears from our present results to be, at the one extreme unlikely and at the other unproven. Further, since there is evidence (Stearn, 1930) that under certain conditions some acidic proteins and acidic lipoids may react positively (and the weakening of the P.A.S. reaction after bile-extraction may be significant in this context), it is by no means unlikely that substances of this kind are concerned in the Gram reaction of the basiphil granules. The work now in progress is, in part, designed to test such an hypothesis.

We should like to acknowledge a gift of ribonuclease from Prof. A. Haddow, Director of the Chester Beatty Research Institute, London.

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The Cell-theory: A Restatement, History, and Critique

Part III. The Cell as a Morphological Unit

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SUMMARY

A long time elapsed after the discovery of cells before they came to be generally regarded as morphological units. As a first step it was necessary to show that the cell-walls of plants were double and that cells could therefore be separated. The earliest advances in this direction were made by Treviranus (1805) and Link (1807).

The idea of a cell was very imperfect, however, so long as attention was concentrated on its wall. The first person who stated clearly that the cell-wall is not a necessary constituent was Leydig (1857). Subsequently the cell came to be regarded as a naked mass of protoplasm with a nucleus, and to this unit the name of *protoplast* was given. The true nature of the limiting membrane of the protoplast was discovered by Overton (1895).

The plasmodesmata or connective strands that sometimes connect cells were probably first seen by Hartig, in sieve-plates (1837). They are best regarded from the point of view of their functions in particular cases. They do not provide evidence for the view that the whole of a multicellular organism is basically a protoplasmic unit.

Two or more nuclei in a continuous mass of protoplasm appear to have been seen for the first time in 1802, by Bauer. That an organism may consist wholly of a syncytium was discovered in 1860, in the Mycetozoa. The syncytial nature of the Siphonales was not revealed until 1879. The existence of syncytia constitutes an exception to the cell-theory. No wholly syncytial plant or animal reaches a high degree of organization.

Natural polyploidy was discovered by Boveri (1887), who was also the first to produce it experimentally (1903). Although many organisms contain some polyploid constituents and others are polyploid throughout their somatic tissues, yet diploid and haploid protoplasts (haplocytes and diplocytes) are the primary components of plants and animals and are still retained as such by most organisms. The haplocyte is more evidently unitary than the diplocyte.

Haplocytes and diplocytes are not composed of lesser homologous units, and with the necessary reservations required by the existence of syncytial and polyploid masses of protoplasm, they may therefore be said to be the fundamental morphological units of organisms.

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Contents

Introduction

In the first of this series of papers (1948) the cell-theory was restated in the form of seven propositions. The second of these was as follows:

Cells have certain definable characters. These characters show that cells (*a*) *are all of essentially the same nature and* (*b*) *are* units *of structure.*

Part II of the series (1949) was devoted to the statement labelled (*a*) in this proposition; that is to say, to the fundamental similarity of all cells as revealed by the discovery of protoplasm and the nucleus. We are now concerned with the part of the proposition labelled (*b*); that is, with the idea of the cell as a morphological unit. The idea of the cell as a functional unit cannot escape mention here, but this aspect of our problem will be more fully considered under the heading of Proposition V.

Early Researches Bearing on the Morphological Separateness of Cells

The cellular nature of plants is much more obvious than that of animals, and the earliest cytological observations were naturally made mostly on plants. Since it was the cell-wall and not the cell itself that called attention to the existence of cellular structure, attention was concentrated on the wall as a matter of course. The wall that separates two cells appears single on superficial examination, and the early observers were not inclined to regard cell-walls as separate boxes enclosing material within. The idea of a cell as a morphological unit only originated when it was found that the wall separating two cells was in some cases demonstrably double. The history of this advance will now be related.

It has been mentioned in Part I that Grew described the parenchyma of plants as 'nothing else but a Mass of Bubbles' (1672, p. 79). In his later work he formed a wrong impression and made his well-known comparison with lace. This false simile had a profound influence that remains with us today in such erroneous names as *tissue* (and its counterparts in other languages) and *histology*. We are all accustomed today to speaking of the 'tissues' of the body and may tend to forget that until relatively recent times this word meant

nothing else than a textile fabric woven of threads. Until late in the seventeenth century no one could have conceived how such a name could be applied to a part of the body of an organism, and indeed the word was not used in this sense in literary English until about 120 years ago. It is strange to reflect that the modern English usage derives indirectly from a fallacy about the microscopical structure of plants published by Grew in 1682.

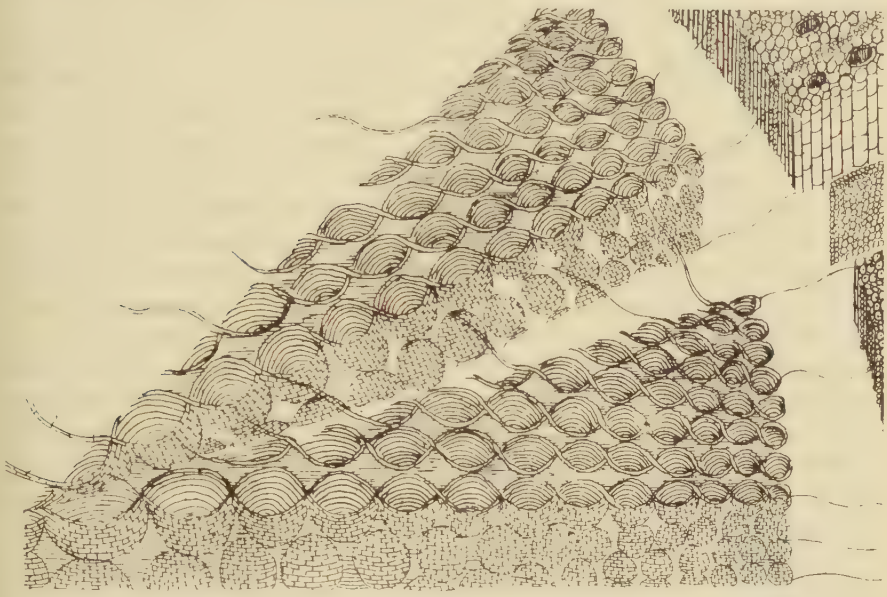

FIG. 1. Part of Grew's drawing of a piece of a branch of the sumach, highly magnified. The supposed fibres, corresponding to the threads of lace, are well shown. (Enlarged from Grew, 1682, plate 40.)

Grew considered that all the parenchymatous parts of a plant, including its fruit, consisted of 'Threds or Fibres', variously woven together. In a well-known passage (1682, pp. 121–2) he compares these fibres to the threads of lace. The comparison is actually to lace while it is being made in a horizontal piece upon a cushion. The pins, inserted vertically into the latter, are imagined to be hollow, and thus represent the vessels of the wood. The lace is supposed to be made in many thousands of layers, one on top of another. The holes between the threads represent the cavities of the cells; and it is understandable that the layers of threads could be added in such a way as to make closed vesicles instead of holes. 'And this', remarks Grew, 'is the true Texture of a *Plant*: and the *general composure*, not only of a *Branch*, but of all other *Parts* from the *Seed* to the *Seed*.'

Of Grew's many figures, the one that illustrates best the ideas just expressed is his representation in perspective of a piece of a branch of the sumach, highly magnified. This figure is here reproduced as fig. 1. It shows clearly his belief in the essentially fibrous nature of plant parenchyma. It follows

from what Grew writes and also from this illustration, that if there is a unit in plant tissue, that unit is a fibre and not a cell; for the latter is merely a space left here and there by the intertwining of the fibres.

For well over a century after the time of Grew, attention continued to be focused mainly on the cell-wall rather than on the contents of the cell. The belief that the wall consisted of microscopically-visible fibres did not persist (though the nomenclature based on that mistake was retained); but another kind of error began to be generally accepted. It was thought that the system of cell-walls was a continuous substance throughout the whole of a plant. Spaces (cells and vessels) were supposed to appear in this continuous substance; nourishment was thought to flow through them to the really essential constituent, the continuous membrane or cell-wall. The cells were often regarded as freely open to one another. According to this belief in its extreme form, a plant would consist of two substances, a membranous meshwork and the nourishing fluid filling its meshes; each would be completely continuous. There would indeed be cells, or enlarged meshes; but there would be no unit of structure.

One of the most obstinate adherents to this view was Brisseau-Mirbel, who wrote (1808, pp. 14 and 128): 'The first idea, the fundamental idea is that all vegetable organization is formed by *one and the same membranous tissue*, variously modified. This fact is the base of all the others. The contrary idea is a source of errors. . . . Plants are composed of cells, all the parts of which are continuous among themselves; they present only one and the same membranous tissue.'

In very early times the contrary opinion began to appear, though the development of two opposing schools of thought came slowly, and more than a century was to elapse after the publication of Grew's *Anatomy of Plants* before anyone began to think clearly in terms of the cell as a unit. Grew's contemporary, Malpighi, nevertheless inclined towards what may be called the utricular view: he did not regard the cell as merely a space between interlacing fibres. This appears, for instance, in the account of the microscopical structure of the petals of the tulip and other plants, in his *Anatome plantarum* (1675, pp. 46–47). He remarks that when the surface is torn, the outflowing material contains microscopical objects resembling icicles in shape, which had been entangled loosely together in the intact petal. Each is formed, he tells us, of utricles arranged in a row. One of his illustrations of such a row is reproduced here as fig. 2. There is nothing in such descriptions that would call to mind the lacework of Grew.

FIG. 2. Malpighi's drawing of a row of cells from the petal of the tulip. (Malpighi, 1675, plate 28, fig. 164.)

The utricular view had its supporters in the succeeding century. Duhamel du Monceau (1758) made some suggestive observations. He separated by maceration small pieces of the 'tissu cellulaire' of the branches of the lime

tree. Sometimes he succeeded in detaching little oval bodies of fairly regular shape, which he thought might be the 'vésicules' of Malpighi and Grew; but neither his words nor his illustration (fig. 7 on his plate 2) permit us to feel any more certain of this than he did himself.

Before the cell could be regarded as a unit it was necessary to show that the wall between two contiguous cells was double and that the cell could therefore be isolated as a separate object. This separation can be achieved because of the relative softness or solubility of the pectose or pectic acid of the middle lamella. The first person who clearly demonstrated that plant-cells are separable units was G. R. Treviranus. Referring to the globules mentioned by Wolff (see Part I of this series), Treviranus writes (1805, p. 233), 'I have nowhere seen these little bladders so clearly as in the buds of Ranunculus Ficaria L. A thin section of this, brought under the magnifying-glass in water, allows itself to be divided by the point of a needle into nothing but little bladders (in lauter Bläschen).' He generalizes thus: 'The first beginning of all organization of the living being is an aggregation of little bladders that have no connexion with one another. From these arise all living bodies, just as they are all dissolved into them again.' This statement, which resembles Oken's speculations as expressed in *Die Zeugung* (1805), is of course an induction based on insufficient evidence; but Treviranus's conception of the cell as a unit had actual observation behind it.

Shortly afterwards Link (1807) made a considerable advance towards the understanding of the separateness of cells. He remarks (p. 11) that most authors suppose the existence of an open communication between them, so that the 'Saft' of one can pass into another. Link denies this. When he put cut twigs in coloured fluids, he never observed the passage of the fluid from one cell to another, except when a cell-wall happened to be damaged. He also noticed that certain plant cells have red sap, but are surrounded by others that are uncoloured. To him, cells *and their walls* were separate. His remark on this subject is of particular interest. 'At places where cells adjoin one another', he writes (p. 13), 'one often notices a double line, as if there were a space between the cells.' He illustrates this by a drawing, here reproduced as fig. 3.

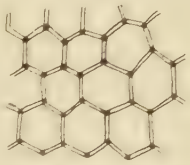

FIG. 3. Link's drawing of a transverse section through the pith of *Datura tatula*, showing the double line where each cell abuts on another. (Link, 1807, plate 1, fig. 2.)

Two years later Link (1809) was quite definite on this subject. 'Cellular tissue', he writes (p. 1), 'consists of little bladders completely separated from one another; but their membranes [cell-walls] usually lie so close to one another that they appear to constitute only a single partition-wall.' He instances the petioles of the large leaves of *Rheum undulatum* and other species as suitable objects for exhibiting the separateness of the cell-walls. The petioles of certain ferns (he mentions *Scolopendrium vulgare* and *Adiantium pedatum*) provide striking examples.

Link continued his investigation (1812) by studying boiled plant tissues, such as kidney-beans and the roots of several garden-plants. He found that not only the cells of the parenchyma but also the elongated bast-cells are separated by this treatment or become separate if gentle pressure is subsequently applied. Link also found completely separate cells, each with its own wall, in many ripe fruits, especially berries. He considered all partitions between cells as originally double; often they remain so and the double wall can be seen, while in other cases it becomes single by subsequent fusion.

Meanwhile L. C. Treviranus (1811) had arrived at similar results. He found that in some cases mere sectioning of plant tissue sufficed to make the cells fall apart, and that this separation can be promoted by pulling gently on the tissue. He reached this conclusion (p. 1): 'It is at once evident to every unprejudiced observer that the cellular tissue of plants is an aggregate of semitransparent bladders, which cohere to a certain extent.' He attacked the contrary opinions of Grew and Brisseau-Mirbel.

Moldenhawer (1812) tried macerating sections of plant tissue in water. He expressed his results very clearly. He remarks on the double nature of the cell-wall.

> But maceration [he writes (pp. 81, 86)], if only employed with due caution, also splits the cellular substance into separate, self-contained utricles . . . [the cellular substance] breaks down sooner or later, according to the firmness of the connexion, into single closed utricles that show no trace whatever of injury, which they necessarily would reveal in the form of irregularly-broken, jagged walls, if there were violent rupture of one and the same continuous tissue. . . . Such an aggregate of single cells has nothing in common with a tissue (Gewebe), and the name *cell-tissue* (Zellgewebe) therefore appears to be less appropriate than that of *cellular substance*, that is, substance consisting of cell-shaped utricles.

Like Link, Moldenhawer mentions cells with differently coloured sap lying close to one another. He explains Grew's 'Threds or Fibres' as mere wrinkles in cell-walls.

Dutrochet (1837) introduced the use of concentrated nitric acid, in a tube immersed in boiling water, as a macerating agent. He showed by this means that the cell-wall is double, and that the substance of plants can be separated into its constituent elements or cells.

By this time, however, the older view had lost its grip: even Brisseau-Mirbel admitted his error. In his work on the liver-wort *Marchantia* he writes of 'the utricular composition of the tissue, which I formerly denied, and of which today I confess the reality' (1835, p. 352). He thus allowed that the cavities of the utricles were not in free communication with one another; but he still adhered to the singleness and continuity of cell-walls. He communicated his paper on the subject to the Académie des Sciences in 1831, but it was not published till four years later. In the meantime his conversion had been complete, and he announced it very frankly in a note appended to the paper.

The cellular tissue of *Marchantia polymorpha* [he writes (p. 363)] did not offer me spaces between cells. These canals, which are nothing else than the spaces the utricles leave between them, and which for this reason M. Tréviranus calls intercellular, exist in many plants and are absent in others. Thus one can say that the utricles composing cellular tissue are welded together either completely or incompletely. . . . today, when I have obtained the most direct proof of the utricular composition of the tissue, I understand and I see the spaces, which I neither understood nor saw before, and I retract my objections to the fine discovery of M. Tréviranus.

These magnanimous words may be said to mark the end of the controversy about the morphological separateness of ordinary plant-cells.

The Discovery of the Cell-membrane

A clear picture of the nature of the cell could not be obtained so long as the wall was regarded as an essential part. It was necessary to realize that the wall was sometimes present and sometimes absent, while the cell itself was always bounded by a special *membrane*, not mechanically separable from the ground-cytoplasm within. This advance could not be made in one step. It was necessary first to discard the cell-wall as unessential to the idea of a cell, which was then looked upon as consisting of 'naked' protoplasm. The discovery of the cell-membrane came much later.

It must be remarked at the outset that the early workers generally called the cell-wall the *membrane* or *Membran*. This rather confusing usage will appear in various passages quoted in the present paper.

Most cells of animals are obviously devoid of a covering corresponding to the cellulose cell-wall of plants, but one can understand why the cytologists of Schwann's time did not recognize this. It was partly because they were swept away by the new idea of the 'Uebereinstimmung' of all cells, whether plant or animal, and this correspondence would be greatly weakened if one of the most characteristic features of plant cells were found to be absent from those of animals. They therefore looked with confidence for a cell-wall in the animal cell, and in many cases found what they were seeking. The free border of intestinal epithelial cells, the vitelline membrane of eggs, and the cortical layer of ciliates are examples of real structures that seemed to them to represent the cell-wall. Such walls, however, were also described where in fact there are none. This was probably due to the appearance of double lines at the edges of cells, caused by the low numerical aperture of the microscopical objectives available at the time.

The study of animal embryos might have caused a change of opinion, because the blastomeres, which clearly represent the whole organism in its early stages, are devoid of anything resembling the cell-wall of plants; but if blastomeres were to be regarded as cells, as was being suggested, then a cell-wall must be shown to exist, according to the opinion of the day. Reichert (1841), who had the help of du Bois-Reymond, investigated this matter in the developing eggs of amphibians. They isolated uninjured blastomeres in

late cleavage-stages and placed them in distilled water under the microscope. According to their account, a surface-membrane was pushed off by endosmosis. They homologized it with the familiar cell-wall and regarded it as evidence for the cellular nature of blastomeres.

Ecker reported that he had seen movements in the blastomere of the frog; he thought these were inconsistent with the presence of a cell-wall, and therefore with the cellular nature of blastomeres (see Remak, 1851). It was Ecker's report that brought the great embryologist, Remak, into the controversy, unfortunately on the wrong side. He opposed Ecker's opinion, and claimed to see two firm membranes surrounding each of the upper blastomeres in the eight-cell stage of the frog; he regarded this as evidence that blastomeres were cells (Remak, 1851). Like Reichert, he saw the membrane surrounding the blastomeres swollen by the osmotic absorption of water, and might have realized the existence of a cell-membrane not corresponding to the cell-wall of plants. He was impressed, however, by the detached envelope that he saw round blastomeres that had been treated with various reagents (hydrochloric, sulphuric, and chromic acids, mercuric chloride, and alcohol), and convinced himself that this envelope corresponded to the cell-wall of plant tissues (1855, pp. 135–6, 173–4). He opposed the view that the surface of a blastomere was merely a modified part of the protoplasm. The cell-walls of plants could be exhibited and distinguished from the underlying protoplasm by simple techniques already in common use in his time, and he looked forward eagerly to the discovery of methods that would play the same part in animal cytology, with equal clarity and certainty.

The first person who stated in unequivocal terms that the cell-wall is not a necessary constituent of the cell was Leydig. He wrote (1857, p. 9):

> . . . not all cells are of bladder-like nature; a membrane separable from the contents is not always distinguishable. For the morphological idea of a cell one requires a more or less soft substance, primitively approaching a sphere in shape, and containing a central body called a kernel (*nucleus*). The cell-substance often hardens to a more or less independent boundary-layer or membrane, and the cell then resolves itself, according to the terminology of scholars, into *membrane*, *cell-contents*, and *kernel*.

The idea that a primitive cell is devoid of a wall was recognized by de Bary in the first of his important contributions to the knowledge of Mycetozoa (1860, p. 161). He wrote of the flagellulae that have emerged from spores: 'In the swarmers there is no cell-membrane in the ordinary sense of the term, but there is indeed a nucleus. As has already been shown above, they are to be regarded as skinless or primordial cells. . . .'

Max Schultze (1860), who had studied protoplasm in various Protozoa, especially the Foraminifera, and had confirmed some of de Bary's work on Mycetozoa, made the following generalization (p. 299): 'But the less perfectly the surface of the protoplasm is hardened to a membrane, the nearer to the primitive *membraneless* condition does the cell find itself, a condition *in which*

it exhibits only a small naked lump of protoplasm with nucleus. . . .' Later in the same paper (p. 305) he repeats his definition of a cell as 'ein nacktes Protoplasmaklümpchen mit Kern'.

Schultze now turned to the study of striated muscle and produced the paper (1861) that is so commonly quoted in textbooks of the history of biology. The writers of these, however, have overlooked the rather peculiar character of Schultze's communication. He had set himself the problem of discovering the nature of the 'Muskelkörperchen' or small masses of protoplasm containing nuclei that occur among or outside the contractile elements of striated muscle. He reached the remarkable conclusion that each is to be regarded as a cell. It follows that in the fully differentiated muscle-fibre the contractile elements are extracellular. He knew that each little mass of protoplasm has no cell-wall surrounding it, and his particular point is that this absence of a wall does not indicate that the Muskelkörperchen is not a cell. This led him on to consider what are the essential characters of a cell. He was not content with the definition that had prevailed in the past: 'a vesicular structure with membrane, contents, and nucleus.' To find out what was essential he turned to blastomeres. 'From what has gone before', he wrote (p. 11), 'the component parts of the blastomeres are *nucleus* and *protoplasm*, and our definition of what one has to call a cell assumes the following form: *a cell is a little lump of protoplasm, in the interior of which lies a nucleus.*' The actual words are: 'Eine Zelle ist ein Klümpchen Protoplasma, in dessen Innerem ein Kern liegt.' Schultze refers in a footnote to Leydig's definition. In another place (p. 9) he defines cells as 'little sheathless lumps of protoplasm with nucleus'. He says that the protoplasm holds together because it does not mix with water. 'A membrane', he insists, 'is not necessarily connected with the idea of a cell.' He even considers it a sign of degeneration. 'A cell with a membrane differing chemically from protoplasm is like an encysted infusorium—like an imprisoned monster.' Schultze recognizes that his definition cannot be wholly reconciled with the word *cell*, which conveys the idea of something provided with a distinct wall.

Brücke (1862) agreed with Schultze that the wall is not a necessary attribute of animal cells, but Remak (1862) repeated and amplified his old conclusions. He insisted that the animal cell has at its surface a 'Hülle' or 'Membran', chemically distinct from the ground cytoplasm; and he mentioned once more the separation of this envelope from the underlying protoplasm by chemical agents. He considered that in animals, as in plants, cell-division occurs by the ingrowth of solid septa from the envelope into the protoplasm. Schultze, however, had the support of de Bary in the latter's monograph on Mycetozoa (1864). 'The skin of the cell', wrote de Bary (p. 106), 'is therefore no essential attribute of the cell: it may be formed, but need not.'

A notable advance had been made when the cell came to be regarded as a lump of naked protoplasm with a nucleus, even though the existence of the cell-membrane was not yet clearly recognized. The advance demanded a change in nomenclature, for a cell is a box and a lump of protoplasm is not.

To no one did the old name seem so absurd as to Sachs. Hooke had named the cells of plants after the cells of the comb in a beehive; and if it were right to call the protoplasmic unit a cell, then, according to Sachs (1892, p. 60), a bee should be called a cell, and the cell of the comb should be called the capsule of the cell! Hanstein had been thinking along these lines long before. He recognized that such degree of independence and functional individuality as the cell possesses reside in its protoplasm. This word, however, conveys no sense of an object, but only of the material of which an object may consist. He therefore coined the name 'Protoplast' for the protoplasmic part of a single cell (1880, p. 169). He applied the name to the vital units of both plants and animals. The unit might secrete a wall, but he recognized that this was far from being necessary; for the protoplasts of many animals had no external covering (p. 217). Thus Hanstein agreed in general with Leydig, Schultze, Brücke, and de Bary, but expressed his ideas more exactly. The word protoplast is useful, and modern cytological writings would benefit from a more frequent use of it.

It gradually became apparent that if the vital unit were indeed naked, yet it might at least have a skin. The early comments on this subject are equivocal: we cannot tell whether the authors were referring to the special outer layer or ectoplasm exhibited by certain protoplasts, or to the *membrane* that is always present. Hanstein himself remarked on the skin-like, firmer, 'äussere Hautschicht' of the protoplasm of the plant-cell (1880, p. 167 and fig. 1); he called the Tonoplast the 'innere Hautschicht'. This was a considerable advance beyond the unqualified idea of naked protoplasm, but Hanstein did not undertake the physiological studies that would have been necessary to disclose the real nature of the membrane. The swelling and shrinkage of cells by osmotic pressure might have led to its recognition had not the early work on this subject been confined to the vacuolated plant cell. Pringsheim (1854, p. 51) noticed that solutions of salts, acids, and sugar caused the 'Zellinhalt' to collapse inwards away from the cell-wall. In his famous study of this subject de Vries (1884) defined plasmolysis as the detachment of the living protoplasm from the cell-wall (Zellhaut) through the action of aqueous solutions. He used various plant cells, but chiefly the violet epidermal cells of the lower surface of the leaf of *Tradescantia discolor*. He realized that the membrane responsible for the osmotic phenomena was at the boundary of the vacuole, and since it was involved in the maintenance of turgor, he named it the Tonoplast (1884; see also 1885, p. 469).

Osmotic studies of animal cells would have given new insight into the nature of the boundary of living protoplasm, because the swelling and shrinkage of cells that lacked a vacuole would have directed attention to the true cell-membrane; but such studies were not undertaken at the time, and the view expressed by Leydig and the others prevailed. For more than thirty years it was generally agreed that in its most primitive form the cell consisted of naked ground-cytoplasm (with a nucleus). It required the genius of Overton to recognize a clear distinction between the ground-cytoplasm and cell-

membrane, and to bring forward strong evidence that the latter was always present as a covering. He had spent a number of years in a study of the osmotic properties of the living cells of plants and animals. It was known that an 8 per cent. cane-sugar solution caused slight but definite plasmolysis in *Spirogyra*. Overton (1895) tried a solution of ethyl alcohol of the same osmotic pressure, and found no plasmolysis. He thought it possible that this might be due to easy penetration of the outer part of the protoplasm by alcohol. He extended his observations to many diverse plant-cells, with the same result. He then discovered that a number of other substances (various alcohols, ethers, acetone, aniline, phenol) exerted no plasmolytic effect. He realized that it was not the cellulose cell-wall but the 'Grenzschicht' of the protoplasm that was responsible for osmotic effects. He found that in general animal cells resemble those of plants in admitting certain particular kinds of substances and excluding others. On the basis of these observations he produced the diagram of the plant-cell here shown as fig. 4. The diagram makes a clear distinction between ground-cytoplasm, cell-membrane, and cell-wall. Hanstein's diagram (1880, fig. 1) is comparable, but it was not based on a clear understanding of the remarkable properties of the cell-membrane.

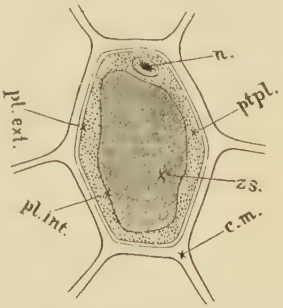

FIG. 4. Overton's diagram of a typical plant-cell, showing the distinction between the cellulose cell-wall (c.m.) and the cell-membrane (pl. ext.). (Overton, 1895, fig. 1.)

Although Overton's diagram marks an important advance, the kind of evidence on which it was based was not quite so satisfactory as an ocular demonstration would have been. A distinction must be drawn between what can be seen and what inferred on indirect evidence. In making his diagram Overton had to decide arbitrarily what thickness he would ascribe to the boundary-layer of the protoplasm.

Overton noticed that the substances that enter cells easily (and hence do not cause plasmolysis) are those that are more soluble in ether, fatty oils, and similar substances than in water, while those that have difficulty in entering cells are those that are readily soluble in water but scarcely or not at all in ether and oil. He was thus driven to the conclusion that the outer layer (Grenzschicht) of the cell must be impregnated with a substance that has dissolving properties similar to those of fatty oils. He rejected the possibility that the substance could be a triglyceride, because he found that filamentous algae could be kept without damage in a solution of sodium bicarbonate that would have saponified a fat. He thought cholesterol or a cholesteryl-ester, with perhaps lecithin and sometimes triglycerides in addition, as the most probable composition of the impregnating substance (Overton, 1899).

So far, Overton had relied on the absence of plasmolysis as evidence that certain substances had entered cells. He now sought more direct indications by following the behaviour of coloured substances (1900). He studied the

capacity of various dyes to enter cells, and related it to their solubility in lipoids. He found in general that basic dyes are soluble in melted cholesterol and in solutions of lecithin in organic solvents, and enter living cells easily, while acid dyes are insoluble and do not enter. The exceptions proved the rule, for methylene blue tannate, though basic, is almost insoluble in the solvents mentioned and is not taken up by living cells, while the acid dyes, methyl orange and tropaeolin, are somewhat soluble and are taken up slowly. Overton thus proved the connexion between lipoid-solubility and the capacity of substances to enter living cells, and strengthened the evidence he had previously obtained for the existence of a special lipoid-containing membrane on the surface of cells.

Very various methods have been adopted by later students of the cell-membrane. More refined investigations of permeability have been made; the tension at the surface has been measured; the capacity of various agents to destroy the membrane has been studied; approximations to its thickness have been obtained by indirect methods. The results of these and other experimental studies have been brought together in theoretical diagrams of the molecular and ionic structure of the membrane. The researches on this subject have been admirably reviewed by two active workers in the field (Davson and Danielli, 1943; Danielli, 1951). It suffices for the present purpose to say that although much has been discovered, nothing has occurred to shake the foundations laid by Overton. His work has been of great importance for the cell-theory. Nothing can be a unit that has not a distinguishable boundary. Thanks to Overton we know where that boundary is: we can say with confidence, in many cases, what is part of the protoplast and what is external to it.

Connective Strands Between Cells

There are several theoretical possibilities as to the nature of the connective strands that are sometimes seen to extend from one cell to another. These are illustrated in fig. 5. In A and B the connexion is made solely through the cell-wall. In C the strand consists of the material of the cell-membrane, while in D and E the ground cytoplasm participates, though the membranes, double (D) or fused (E), still to some extent separate the cells. In F there is direct continuity between the ground cytoplasm of the two cells; and though there may be no cyclosis involving the passage of protoplasm from one cell to the other, yet there is obviously an easy route for the diffusion of molecules and ions. It is often impossible to decide which kind of strand is present in particular cases. The subject is difficult from the technical standpoint: fixed preparations are liable to be misleading. As Weiss has pointed out (1940, p. 35), if a preparation shows connexions between cells, we often cannot be sure that they are not formed of coagulated intercellular matter; while if it does not, there may have been connexions in life that were broken by fixation.

Connexions involving the cell-membrane or ground-cytoplasm are often called cell-bridges; but a bridge is something across which there is movement, and it would be begging an important question to give them a general name

that implied the transport of material from one cell to another. The non-committal name of Plasmodesmen (singular Plasmodesma), introduced by Strasburger (1901, pp. 503, 607), is preferable. The Greek plural plasmodesmata will be substituted here for the German, as being better suited for international use. It is convenient to employ it in a wide sense, to cover all the possible arrangements shown diagrammatically in fig. 5, C–F. It should be mentioned that the most active study of plasmodesmata took place during

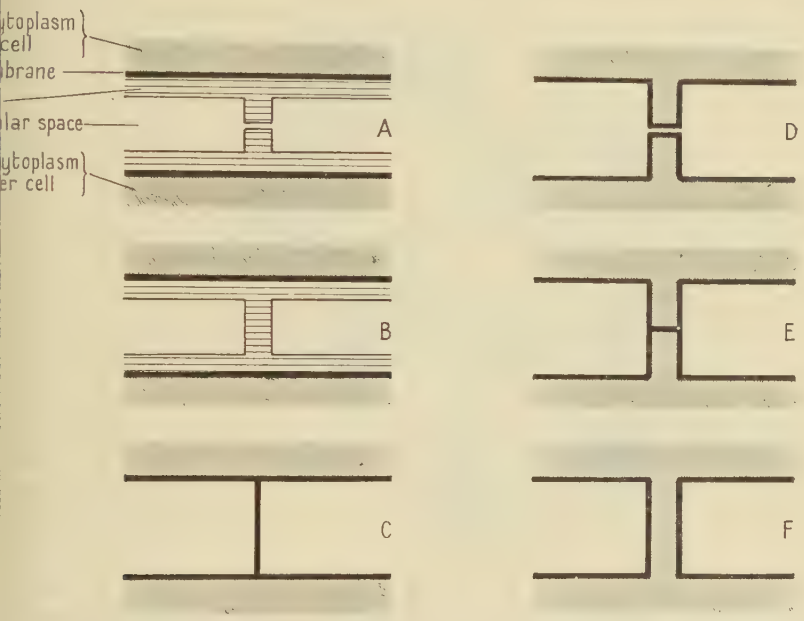

FIG. 5. Theoretical possibilities as to the nature of connective strands between cells.

the long period when cells were commonly regarded as 'naked' lumps of protoplasm, before Overton had proved the existence of the cell-membrane: nice distinctions such as those shown in fig. 5, C–F, would not have had much meaning at that time.

Plasmodesmata must have been seen first in the sieve-plates of plants, which were discovered by Hartig in 1837 (see Esau, 1939) and later re-investigated by him (1854). At first their real nature could not be appreciated because biologists had not yet recognized the nature of protoplasm itself (see Part II of the present series of papers). Sachs, however, who studied them in *Dahlia* (1863), clearly recognized their character, for he mentions that the apertures of the sieve-plates are filled with protoplasm, which he stained with iodine. Modern research shows that two adjacent sieve-tubes are separated by a layer of a substance containing phospholipine, where they abut on one another in the apertures of the sieve-plate; and in some cases there are two layers of the lipoid material, with a very narrow space between (Salmon, 1946, p. 77 and fig. 11). If the lipoid represents a thickened cell-membrane,

the plasmodesmata connecting sieve-tubes are of the types represented diagrammatically in fig. 5, D and E.

After the discovery of the plasmodesmata of sieve-tubes, many years elapsed before anything of the kind was shown to exist in other plant-cells. It is stated by Goebel (1926, p. 118) that Hofmeister demonstrated connexions between the cells of the endosperm of *Phytelephas* and *Raphia* in the course of a lecture given during the winter of 1873–4; but nothing was

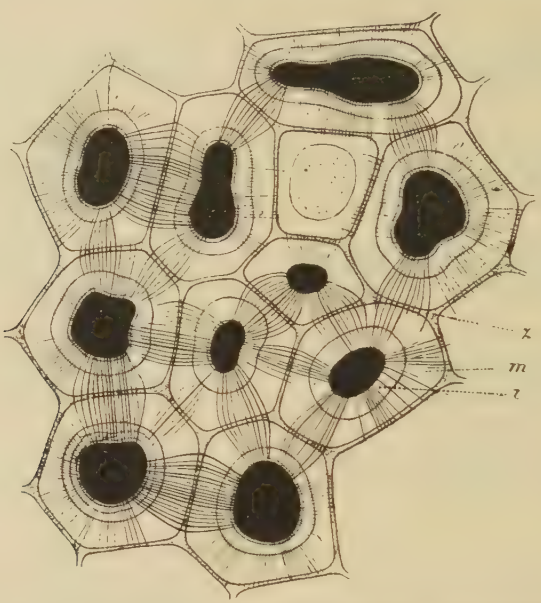

FIG. 6. Tangl's figure showing plasmodesmata in the endosperm of *Strychnos nux-vomica*. The protoplasm (including that of the plasmodesmata) has been stained with iodine-iodide. *i*, inner, and *m*, outer part of the cell-wall; *z*, material lying between adjacent cell-walls. This figure (with those accompanying it in Tangl's paper) was the earliest representation of any plasmodesmata other than the strands connecting sieve-tubes. (Tangl, 1879–81, plate 5, fig. 10.)

published. Fromman (1879), who had come to the conclusion that the protoplasm of the ganglion-cells of the mammalian retina is continuous from cell to cell, turned to plants in search of similar connexions and claimed to find them among the epidermis and parenchyma of the leaves of *Rhododendron* and *Dracaena*. Tangl (1879–81) discovered plasmodesmata between the cells of the endosperm of *Strychnos nux-vomica* and of palms (*Areca oleracea* and *Phoenix dactylifera*). He showed by staining with iodine that the protoplasm was continuous between neighbouring cells through narrow canals traversing the cell-membrane (fig. 6), and considered that the cells entered into a unity of a higher order through these connexions. He thought that there was a striking similarity between the plasmodesmata of endosperm and the (supposed) fibres of the remnant of the mitotic spindle (p. 182). Elsberg (1883) copied the use of silver nitrate and gold chloride from animal histology, and

by the use of these reagents claimed to find connexions between various cells of flowering plants. Russow (1884), who had already studied sieve-plates (1881) and was acquainted with Tangl's discovery, announced the existence of intercellular (non-nucleated) protoplasm in the medullary rays of *Acer*, and said that it was connected by strands with cellular protoplasm. He went farther and claimed that 'in every plant throughout its life the whole of the protoplasm stands in continuity' (p. 581). Gardiner (1884) also thought the existence of protoplasmic filaments connecting cells by no means uncommon: the wide perforations in sieve-plates he regarded merely as special cases of a quite usual arrangement. Kienitz-Gerloff (1891*a*) made a general study of the subject, and reported the existence of plasmodesmata in mosses, ferns, horse-tails, and conifers, as well as in mono- and dicotyledons. He came to the conclusion that the whole of the living material of the higher plants is bound together by connexions, though he admitted (p. 23) that in many cases he could not find them. He examined them carefully in the tissues of *Viscum*, where they are particularly evident, and showed that they are not related to the spindle, as this disappears completely before they are formed (1891*b*). Kuhla (1900), like Kienitz-Gerloff, found the mistletoe particularly suitable for investigations of the subject. He considered that all living cells were connected by plasmodesmata. Davis (1905), in a useful general discussion of the whole problem, pointed out that plasmodesmata arise in some cases by incomplete cell-division, in others by outgrowth from previously separate cells. Typical plasmodesmata, in his view, arise in the latter way and are to be regarded as differentiations of the cell-membrane only (pp. 224–6).

Plasmodesmata of particular interest exist in cycads, where they were discovered by Goroschankin (1883), connecting the 'Corpusculum', as he called it, with the surrounding jacket-cells of the endosperm. It is unfortunate that this particular cell appears not to have a generally recognized name; for it cannot properly be called the ovum until its nucleus has divided to form the ovum-nucleus and the ventral-canal-nucleus. It will here be called the developing ovum. Goroschankin found that the cell-wall surrounding it contains a great number of pits, each provided with a sieve-plate through which the protoplasm of the jacket-cells is in open communication with that of the developing ovum. Smith (1904) subsequently discovered the long 'haustoria' connecting the developing ovum of *Zamia*, through pores in its wall, with the jacket-cells. There seems to be no doubt that the haustoria of cycads are nutritive plasmodesmata.

Among the Protista, connective strands between cells are particularly evident in *Volvox*, where they were discovered by Cohn (1875). He described the 'Tüpfelkanäle' or pit-canals in the cell-wall and the delicate thread-like processes that appear to pass through them from one cell to another; he figures these in *V. globator* (in his fig. 1 on plate 2). Strangely enough, Cohn himself thought that there was no actual connexion between the cells, as he supposed that the pit-canals were closed (p. 95); this is curious, because in

his figure he shows obvious plasmodesmata connecting the cytoplasm of adjacent cells. Modern studies (Janet, 1912, p. 48) show that *V. globator* provides one of the clearest instances of the kind of connexion that is illustrated diagrammatically in the present paper in fig. 5F. The reality of protoplasmic communication in *Volvox* was recognized by Bütschli (1883). Meyer (1895, 1896) also regarded the strands as 'Plasmaverbindungen'; he pointed out that they are long and thin in *V. aureus*, but shorter and much thicker in *V. globator*. It is particularly suggestive that the growing macrogamete of *Volvox* is abundantly provided with plasmodesmata connecting it with the surrounding cells (Janet, 1912, p. 91); the arrangement is reminiscent of the developing ovum of cycads.

Among the Metazoa, plasmodesmata were first discovered in the skin of mammals. We may overlook Henle's (1841) description of prickles (Stacheln) projecting from the surface of the cells of the chorioid plexus, partly because there is no certainty whether what he saw were cilia or elements of the striated border of the cells, and partly because it is unlikely that these cells are in fact connected by plasmodesmata. The discovery was made by Weber (1858) in suppurating skin and in epithelial cancers. Weber himself, however, regarded the strands as cilia. They were studied by Schultze (1864*a*) in the lower layers of the epithelium of the mammalian tongue and skin. He made the mistake of thinking that each cell had its own prickles (Stacheln), which interdigitated like the bristles of two brushes pressed together. This opinion he retained in a second communication on the same subject (1864*b*). Schrön (1865) considered that the appearances really indicated the existence of canals in a 'Membran' separating skin-cells. Bizzozero (1876), who appears to have waited six years for the publication of his paper, was the first to describe the plasmodesmata of skin correctly as direct connexions between one cell and another. No one has ever produced evidence that they represent connexions of the type shown in fig. 5F. It seems reasonable to regard them as serving to hold the cells together mechanically, while allowing bending movements.

Similar connexions have been reported from time to time in very various animal cells. Heitzmann (1873) was an enthusiastic student of plasmodesmata. He claimed to display them by the use of silver and gold impregnations in a wide variety of mammalian tissues, including bone-marrow and even cartilage. Kultschitzky (1888) claimed to find protoplasmic connexions between the smooth muscle cells of the intestine, and looked forward to the discovery of a universal system of such connexions between neighbouring cells in both plants and animals. Connexions between notochordal cells, resembling the prickles of skin-cells, were reported by von Ebner (1896).

Reports of direct connexions between nerve-cells were much more plausible because of the undoubted transmission of the nervous impulse from one cell to another. Different opinions were held on this subject from early times, but no strong evidence was forthcoming until the eighties. Using his potassium dichromate and silver nitrate method, Golgi (1883, p. 289) never found

a single anastomosis among the ramifications of the main processes of nerve-cells; but he considered that other processes existed, which subdivided in a complicated manner and anastomosed, so that the nerve-cells lost their individuality and took part in a nervous reticulum. His (1886) was the first to base the contrary view on sound foundations and state it clearly. As a result of his study of human embryos he came to regard the axons of nerve-fibres as outgrowths from separate nerve-cells that push their way between other tissue-constituents; he denied that these outgrowths ever form actual anastomoses (pp. 509, 513). He remarks: 'I present as an established principle, the proposition *that every nerve-fibre arises as an offshoot from one single cell. This cell is its embryonic* (*genetisches*), *nutritive, and functional centre, and other connexions of the fibre are either only indirect, or have originated secondarily*' (p. 513). Shortly afterwards Forel arrived at the same conclusion from his work on the cavy, but expressed it more tentatively: 'I might presume that all fibre-systems and so-called fibre-nets of the nervous system are nothing else than nerve-processes, [each] always [arising] from a particular ganglion-cell' (1887, p. 166). His (1889) went on to describe the outgrowth of the axon from the neuroblast in various vertebrates. The idea of the separateness of nerve-cells has been followed up by many workers, notably Ramón (1934, &c.), in modern times. There are still distinguished neurologists who oppose the neurone-theory and view the nervous system as a reticulum, but some of their arguments are not strong (as, e.g., when Boeke (1940, p. 144) attacks the cell-theory, and the neurone-theory as part of it, on the ground that it 'belonged to the mechanistic and analytic mental attitude' of the nineteenth century). The modern literature referring to the chordates has been well summarized by Nonidez (1944), who reaches the conclusion that nerve-cells are not directly continuous with one another at the synapse.

The nervous system of coelenterates has long been supposed to provide strong evidence for the reticular theory. The belief that the nerve-fibres of these animals are continuous from cell to cell originated with Korotneff (1876), who studied the layer of nerve-cells and fibres that lies below the external epithelium of the acrorhagi ('bourses marginales') of *Actinia*. He described the fibres as running without a break from nerve-cell to nerve-cell. He even thought the fibre maintained its individuality within the nerve-cells. Korotneff mentioned only single rows of cells, but subsequent workers believed that the apparent nerve-net of coelenterates was formed by continuous fibres passing uninterruptedly from cell to cell. This opinion was generally accepted for several decades, though Schäfer had stated in the most definite manner (1878) that each fibre of the bipolar cells of the sub-umbrellar surface of *Aurelia* 'is entirely distinct from, and nowhere structurally continuous with, any other fibre'. He knew that the fibres came into close relation with one another, and thought it reasonable to conclude that nervous impulses passed from one to another, but he considered each nerve-cell with its two processes a separate unit. This was confirmed in modern times by Bozler (1927), who treated the fresh tissues of the jellyfish *Rhizostoma* with reduced

methylene blue solution and exhibited the nerve-cells with their processes as separate units (some bipolar, others multipolar); the units make contact with one another but do not anastomose. He denied, therefore, that the nervous system of *Rhizostoma* is a genuine nerve-net. The results of the physiological study by Pantin (1935, *a* and *b*) of the nervous system of the sea-anemone *Calliactis* are consistent with the belief that here also the nervous system consists of cellular units.

There are, of course, cases in which nerve-cells make such evident junctions that an actual syncytium is formed. Thus in cephalopods each of the two first-order giant-cells, which lie in the brain close to the statocyst, sends back a giant axon, and the two axons are connected by a wide bridge as they pass through the palliovisceral ganglion (Young, 1939). Again, the giant fibre that runs along the ventral nerve-cord of the polychaet *Myxicola* is part of a syncytium, for at least 1,300 nerve-cells are continuous with it, directly or indirectly (Nicol, 1948). These interesting facts, however, throw no light on the nature of ordinary synapses. In the light of existing knowledge it is best to draw the provisional conclusion that the nervous system does not provide us with convincing examples of plasmodesmata, though actual syncytia are found in particular cases.

It was found by Berthold that the developing eggs of the nematomorph worm, *Gordius*, are fixed together like grapes in bunches (see von Siebold, 1843). Reinvestigating the matter, Meissner (1856) reported that the eggs originate in groups of 8–20 in the ovary by bulging outwards from a mother-cell, and remain for a long time in organic connexion with one another through their stalks. It is necessary to mention this, because it would have been the first example of plasmodesmata discovered in animals, if true. It appears, however, from the careful work of Vejdovsky (1888, p. 204), that in reality the eggs are only held together in groups by the ovarian epithelium, which becomes very thin in late stages and closely pressed to the surfaces of the eggs.

The connexion of animal eggs with external protoplasm was first reported by von Ihering (1877) in the lamellibranch *Scrobicularia*, six years before Goroschankin's discovery in cycads. According to von Ihering, the follicles of the ovary are lined by a syncytium. In this, an egg develops by the accumulation of protoplasm round a nucleus. The egg then projects into the cavity of the follicle and eventually remains in connexion with the syncytium only by a narrow stalk. Yolk is seen in the syncytium, in the stalk (where it is arranged in regular lines), and in the egg. Korschelt (1886) undertook a detailed study of the morphological relation between nurse-cells and eggs in various insects. Gross (1901) described and figured the long, narrow 'Dotterstränge' that lead from the nutritive end-chamber of the ovarian tubule of Hemiptera to the oocytes; the end-chamber itself is a mass of protoplasm in which nuclei are degenerating or have degenerated. Later (1903) he studied the relation between nurse-cells and oocytes in other insects. In the carabid beetle *Harpalus* he illustrated a nurse-cell nucleus in

passage along a narrow neck connecting the oocyte with its food-supply (plate 11, fig. 124). In the hemipteran *Triecphora* he described cords like Dotterstränge connecting some of the nurse-cells with the main protoplasmic mass of the end-chamber, which itself supplies the growing oocytes through long Dotterstränge. This arrangement in Hemiptera was confirmed by Mestschershaya (1931), who investigated the permeability of oocytes and end-chamber to various substances in solution, and reached the conclusion that the end-chamber is adapted to take up food-substances and pass them to the oocyte. There seems to be no doubt that the Dotterstränge of certain insects are genuine plasmodesmata of the type shown in fig. 5F (see, e.g., Korschelt, 1936, fig. 38c).

It was reported by Hammar (1896) that in the cleavage of *Echinus* the outermost protoplasmic layer is continuous from one blastomere to the next. Protoplasmic connexions between blastomeres might be thought to indicate a primary condition, characteristic of cells in general, and the subject attracted attention. Flemming (1896) considered that blastomeres are primarily separate but in some cases become secondarily connected by strands. Andrews reported that in the starfish and other echinoderms the blastomeres send out thin protoplasmic processes that join them together (1897), and that some of the cells of the blastulae are connected in this way (1899). Hammar (1897) made a general study of this subject, using as fixative a saturated solution of mercuric chloride in evaporated sea-water, with the deliberate intention of shrinking the cells and thus increasing the intercellular spaces and exhibiting the strands crossing them. He claimed to find protoplasmic connexions between blastomeres in various invertebrates from coelenterates upwards, but it would be unwise to place much reliance on results obtained by a method so particularly liable to produce artificial appearances. In *Dendrocoelum*, as Fulińksi (1916) showed, not only are the blastomeres not connected by plasmodesmata: they do not even touch one another, but lie separately in a fluid derived from the yolk-syncytium.

The belief of Sedgwick in the continuity of protoplasm from cell to cell has been so influential that it is desirable to treat separately the controversy he aroused. In studying the development of *Peripatus* he noticed that the endoderm cells, previously separate, put out branches that anastomosed (1885). This was the origin of his doubts about the truth of the cell-theory. He was later impressed by the structure of the mesenchyme of elasmobranch embryos, which he described as 'a reticulum of a pale non-staining substance holding nuclei at its nodes' (1894). He regarded the ectoderm and endoderm as 'simply parts of this reticulum in which the meshes are closer and the nuclei more numerous and arranged in layers'. What were taken by others to be sites of cell-proliferation were described by him as places where nuclei multiplied. 'In short, if these facts are generally applicable', he wrote, 'embryonic development can no longer be looked upon as being essentially the formation by fission of a number of units from a single primitive unit, and the coordination and modification of these units into a harmonious whole. But

it must rather be regarded as a multiplication of nuclei and a specialization of tracts and vacuoles in a continuous mass of vacuolated protoplasm.' The 'vacuoles' of Sedgwick would be what adherents to the cell-theory would call intercellular spaces not filled by cell-walls or other solid matter. He claimed that nerves were laid down before any trace of nerve-cells could be made out. 'In short, the development of nerves is not an outgrowth of cell-processes from certain central cells, but is a differentiation of a substance which was already in position.'

These arguments were answered by Bourne (1895), who took Sedgwick's objection to the cell-theory to be based essentially on embryological evidence. Bourne himself did not accept the idea that a multicellular organism is actually a 'cell-republic', but he insisted that it is an aggregate of elementary parts, and thought that Sedgwick's views would take us back to the Cytoblastem of Schwann. He considered that in the case of spiral cleavage at any rate the blastomeres are not connected by protoplasmic strands. Sedgwick did not leave Bourne's rather mild criticism unanswered. His study of *Peripatus* had led him to the view that 'the differentiation of the Metazoa had been effected in a continuous multinucleated plasmatic mass, and that the cellular structure had arisen by the special arrangement of the nuclei in reference to the structural changes' (1895). He could not accept the idea of the zygote *dividing* into blastomeres, and he insisted that both ovum and spermatozoon were individuals, simplified so as to make their fusion possible. Animals for him were generally 'tetramorphic': that is, they exhibited four kinds of individual: male, female, spermatozoon, and egg. He thus allowed individuality to the gametes, while denying it to other cells.

Sedgwick's ideas were not novel, for Heitzmann had expressed them long before. In the paper already quoted he wrote (1873, p. 155): '*The animal body as a whole is one lump of protoplasm, in which are embedded to a smaller extent isolated protoplasmic bodies* (wandering bodies, colourless and red blood-corpuscles) *and various other substances that are not alive* (gelatinous and mucous substances in the widest sense, together with fat, pigment granules, &c.).' He compared the whole of a higher animal to an *Amoeba*, and denied that there is any such thing as intercellular substance, even in blood; for him, there was only 'Grundsubstanz' and protoplasm (1873, p. 155). Ten years later, about the time when Sedgwick was first turning his attention to the subject, Heitzmann wrote: 'What have previously been considered as cells prove, in our conception, to be nodal points of a network that traverses the tissues' (1883, p. 57). He applied this generalization to both plants and animals. It was Sedgwick's eminence as a zoologist, however, that gave currency to the new ideas. He spread them not only by his writings but through personal contacts. Dobell, one of the strongest opponents of the cell-theory, was trained in his school.

There is no reason to suppose that protoplasm flows freely through every connexion we may find between cells. Schultze made a comment on this subject long ago that is memorable for its good sense and moderation. 'But

I dispute', he wrote (1861, p. 26), 'that the *individuality* of cell-life is encroached upon by the anastomoses, and I dispute that in normal circumstances, with full integrity of the individual cells, the situation can be even quite roughly interpreted as a protoplasmic vessel-system.' In much the same sense Strasburger (1901, p. 595) drew a distinction between the existence of plasmodesmata on the one hand, and the loss of cellular individuality on the other.

Plasmodesmata should be considered from the functional point of view. Where a necessity has arisen for bulky materials to stream into a particular cell, protoplasmic connexions have evolved for their passage. We have seen examples in the developing ovum of cycads, in the macrogamete of *Volvox*, and in the oocytes of various insects. Where it has been particularly important for cells to combine the property of holding together firmly with that of allowing changes of relative position, strands are seen to pass between neighbouring cells. Flemming (1896) remarked on the physiological necessity for junctions in certain epithelia. Indeed, the mammalian skin provides one of the most familiar examples of plasmodesmata. Another good example is the mesenchyme of the embryos of very diverse animals. In this embryonic connective tissue there are no extracellular fibres, and the function of holding together is served by direct connexions between cells. In some cases we cannot assign a function to the plasmodesmata; but in general we find them serving some particular purpose, and not existing as biological necessities indicative of an essential protoplasmic unity of the whole organism. We have no reason to suppose that cells usually possess them.

Syncytia

The nomenclature of multinucleate masses of protoplasm is very confused. The word *coenocyte* is generally employed in botany to mean a whole plant containing several or many nuclei not marked off from one another by cell-boundaries, but it is also sometimes used for *parts* of plants that contain more than one nucleus in a continuous mass of cytoplasm. The word *syncytium* suffers from having been used in different senses by its originator. Haeckel defined a cell (Cellula) as a lump of protoplasm with a nucleus, and called the expression 'mehrkernige Zelle' a *Contradictio in adjecto* (1866, vol. 1, pp. 275, 296). In coining the word *Syncytium* he restricted its meaning specifically to a complex formed by the fusion of previously separate cells (1872, vol. 1, p. 161); he applied it to the dermal epithelium of calcareous sponges, which he believed to be of this nature. Cienkowsky, however, had invented the word *Plasmodium* for a continuous mass of protoplasm formed by the fusion of previously separate cells (1863*a*, p. 326); he applied the word to a stage in the life-history of Mycetozoa. He knew that this stage was reached by the fusion of nucleate cells, but considered the plasmodium itself to be devoid of nuclei (1863*b*, pp. 435–6). Haeckel accepted this, and thus drew a distinction between a syncytium and a plasmodium (1872, vol. 1, p. 161). Later he supposed that Mycetozoa were at first nucleate and later

non-nucleate; he expressed this by saying that they were syncytia that became plasmodia (1878, p. 51). Later again he evidently revised his ideas on the nature of a *Contradictio in adjecto*, for he referred to the Siphonales and other multinucleate organisms that lacked cell-boundaries as polykaryote cells (1894, p. 70). Finally he stated distinctly that a *Bryopsis* or *Caulerpa* consists of a single cell with many nuclei, and gave a new definition of his word syncytium: 'The whole body consists of a single colossal cell, which includes many nuclei in its voluminous body' (1898, vol. 2, p. 421), He applied the name to Siphonales, Mycetozoa, *Actinosphaerium*, and certain Foraminifera, but not to any constituent part of any organism; and he equated syncytia with plasmodia.

Gegenbaur applied the term syncytium to striated muscle (1874, p. 26); Huxley introduced it into English, with Haeckel's original meaning (1877, p. 113). Delage and Hérouard (1896, p. 41) also restricted the sense of the word to cases in which previously separate cells fuse together.

In an attempt to reduce the confusion caused by these various meanings I shall use the word syncytium to mean *any obviously continuous mass of protoplasm that contains more than one nucleus, whether that mass constitutes a whole organism or part of an organism, and whether the bi- or multinucleate condition has been reached by aggregation of previously separate cells, or by nuclear division without cell-division, or by both aggregation and nuclear division.* I shall restrict the word *plasmodium* to *a syncytium formed by aggregation of previously separate cells, whether subsequent cell-division increases the number of nuclei or not.* The word *coenocyte* appears to be superfluous and will not be used. It may be suggested that if used at all, it should refer to *a syncytium that constitutes a whole organism.*

For the sake of consistency it is necessary to classify as syncytia certain temporary arrangements of nuclei and cytoplasm that are not customarily so regarded. In all cases in which nuclei are formed after mitosis before the cytoplasm has completely divided, a short-lived syncytium may be said to exist. In many (perhaps most) animals the male and female pronuclei do not fuse at fertilization; the two sets of chromosomes only come together at the two-cell stage. We have here an example of a short-lived syncytium with two haploid nuclei. As is well known, the process is carried much farther in some copepods, for double nuclei are sometimes seen up to the 32-blastomere stage, and indications of gonomery may persist even later (Häcker, 1892, 1895; Rückert, 1895).

Cells connected by plasmodesmata should not generally be regarded as constituting a syncytium, because the protoplasm is not obviously continuous. Although, as Fol (1896, p. 211) remarks, there is no sharp distinction between cellular tissue and syncytium, yet doubtful cases are rare. It was pointed out by Pringsheim long ago (1860, pp. 229–30) that the hyphae of certain Saprolegniaceae are constricted at intervals, but not divided right across; usually there is only one nucleus between each constriction and the next. Reinhardt (1892, p. 562) described a similar arrangement in *Peziza* and claimed that

a part of the streaming cytoplasm passed through a central opening in the transverse wall. If so, the 'cells' clearly constitute a syncytium.

Syncytia appear to have been first noticed in plant tissues by Bauer in the style of *Bletia tankervilliae* (Orchidaceae) in 1802. His drawings were not published till much later; the book evidently appeared in sections that were not separately dated (Bauer, 1830–8). In one of the drawings, here reproduced as fig. 7, the loose tissue of the stigmatic canal after fertilization is shown.

FIG. 7. Bauer's drawing of cells in the stigmatic canal of *Bletia tankervilliae*. One 'cellule' contains two and another three nuclei. The drawing was made in 1802. (Bauer, 1830–8, 1st part ('Fructification'), plate 6, fig. 3.)

Bauer noticed that there were from one to three 'specks' in each of the 'cellules'. It seems just possible that the 'cellules' were sections of pollen-tubes. This drawing by Bauer was known to Brown (1833, p. 711), who mentioned it in the famous paper in which he introduced the word *nucleus*. Brown confirmed Bauer's finding and remarked that it was the only example known to him of more than one nucleus in a cell. Meyen (1837, p. 208) reported that two or three nuclei often occur in 'langgestreckten Zellen'. Unger (1841, fig. 6 on plate V) gave a representation of a cell in the root of *Saccharum*, elongating to form part of a vessel; it is in fact a syncytium containing three nuclei. In their botanical textbook Endlicher and Unger (1843, pp. 22–23) remark, like Meyen, that there are often several nuclei in 'langgestreckten Zellen'. Nägeli (1844, p. 62) mentioned the existence of more than one nucleus, without cellular partitions, in pollen-grains, in the pollen-tube, and in the embryo-sack. The more recent literature of syncytia in the vegetative cells of phanerogams has been reviewed by Beer and Arber (1920).

Syncytia were recorded in the gill-cartilages of *Pelobates* and *Rana* by Schwann (1839, p. 23 and fig. 8 on plate 1). It is clear also that Rathke saw the syncytial stage in the development of the crustacean egg. He expresses himself rather obscurely, but this is only to be expected, as the partial cleavage of a centrolecithal egg had never previously been described. In the passage that follows, which is translated from the Latin, he is referring to the cells seen after cleavage. 'In fact', he writes (1844, p. 8), 'before the cells that I have already mentioned originate, there is formed for each of them, among the structural elements of the yolk [i.e. among the yolk-globules], a particular nucleus, consisting of a vesicle filled with a coagulable liquid. As a result of this, every cell now formed is provided with its own nucleus.' Thus Rathke saw many nuclei in the egg before radiating partitions had appeared to divide it (imperfectly) into blastomeres. Kölliker professed to find syncytia in the embryos of certain invertebrates in which cleavage is in fact total, but stated correctly that more than one nucleus occurs in the giant-cells (polykaryocytes) of bone-marrow and in certain nerve-cells (Kölliker, 1852, pp. 20–21 and fig. 7).

It is interesting to trace the course of events as naturalists were groping their way towards the discovery that a whole organism might consist of a continuous mass of protoplasm containing many nuclei. This truth was first revealed by the investigators of the Mycetozoa. It was known to de Bary (1860) that the plasmodium is formed by the fusion of nucleate cells and that each of the chambers of the sclerotium may contain more than one nucleus, but he did not describe nuclei in the plasmodial stage itself. Schultze, however, in the same year described the plasmodia of *Aethalium septicum* as consisting of 'naked lumps of protoplasm, naturally with the nuclei appertaining to them' (1860, p. 301). It is strange that Schultze should have made this discovery, for he undertook no very detailed study of Mycetozoa, and stranger still that some years later Cienkowski (1863, p. 436), as has been mentioned, and de Bary (1864, p. 108) were still of the opinion that the plasmodium lacks nuclei.

Meanwhile events had been leading towards the discovery of the syncytial nature of certain Heliozoa. Many years before, Kölliker (1849) had seen the nuclei of *Actinosphaerium eichhornii*, and described them as 'kern- und zellenartige' bodies (p. 211); but he did not regard them as nuclei. Stein, too, saw them, and remarked that they had 'das Ansehen von Zellenkernen' (1854, pp. 153–4); but he does not pronounce upon the matter. Haeckel thought the objects were probably nuclei, and suggested that bodies within them might be nucleoli (1862, p. 165). Schultze calls them 'zellenartige Körperchen' in one place and 'Kerne' in another; he makes no definite statement as to what they are (1863, pp. 35–36). It was Wallich who first stated definitely that these bodies in *Actinosphaerium* are nuclei; he uses the word 'nucleus', and defines it (1863, pp. 444, 450; see also his fig. 2 on plate X). Two years later Cienkowski (1865) saw and figured several nuclei in another Helizoon, *Nuclearia delicatula*, and recognized their nature. (It should

be remarked that the authors mentioned above called *Actinosphaerium* '*Actinophrys*'.)

In the Radiolaria, also, the numerous nuclei present in certain species were seen long before they were recognized as such. Müller saw them first, in *Acanthometra* (1859, p. 15); he reported the existence of 'many round transparent vesicles' within the central capsule. They were later seen by Haeckel in the Acanthometrida (1862, pp. 141 and 374, and fig. 2 on plate XV), and in the monopylarian *Lithomelissa* (p. 302); he did not know they were nuclei, though he suggested that some Radiolaria might be multinucleate (p. 165). The numerous nuclei of certain Acanthometrida and of *Tridictyopus* (Monopylaria) were for the first time recognized as such by Hertwig (1879, pp. 11, 84), who stained them with carmine. His conclusion was accepted by Haeckel (1887, pp. 32–33).

The syncytial nature of certain other rhizopods was gradually disclosed by the labours of many investigators. Among the thallophytes, on the contrary, most of the important discoveries were made in a short time by one man. Till towards the end of the seventies many of the lower plants were universally regarded as non-nucleate (see, e.g., Haeckel, 1874, p. 409; Sachs, 1874, p. 273; Strasburger, 1876, pp. 86–88; Haeckel, 1878, p. 53). Sporadic discoveries of syncytia had indeed been made. Pringsheim, for instance, as we have seen (p. 178), had described this condition in the Saprolegniaceae, and de Bary (1862, p. 14) had seen stages in the development of the ascus of *Peziza*, with two, four, and eight nuclei not separated by cell-walls. The existence of whole groups of syncytial plants was, however, unsuspected. The plants were known, but their nuclei were not; for though they had been seen in some cases (e.g. in *Cladophora*), they were not regarded as nuclei, simply because there were many of them in each 'cell' (Strasburger, 1876, pp. 86–88 and 324). They were first recognized as such by Schmitz, who revolutionized knowledge of the lower plants by his discoveries. The latter were all published in the journals of local natural history societies. Schmitz used simple methods, staining the nuclei sometimes with alcoholic iodine solution, sometimes with a mixture of haematoxylin, alum, and glycerine after fixation in alcohol or osmium tetroxide solution. He began by studying seaweeds in the Gulf of Athens in 1878, and at Naples in the following year. He reported his first results verbally on 30 November 1878, but there was delay in the printing of this particular paper (Schmitz, 1880*a*). Meanwhile he had published others. He first announced his discovery of nuclei in *Valonia* and related forms, and showed that each of the apparent 'cells' is a syncytium; he founded the group Siphonocladiaceae for these plants, which had up till then been variously classified (1879*a*, 1880*a*). He noted the formation of uninucleate zoospores. Turning next to the Siphonales, he showed the existence of numerous nuclei in the continuous cytoplasm of these non-septate forms (1879*b*). In the same paper he showed the syncytial nature of several Phycomycetes and of the internodes of *Chara*. He also noticed syncytia in parenchyma cells of certain higher plants. He continued his work

and showed that among the Rhodophyceae, the species differ in their nuclear arrangements; in one species the 'cell' may be multinucleate, in another closely related form it may be uninucleate (1880*b*).

The wholly syncytial plants and animals show that the cell, as a morphological unit, is not a necessary component of organisms. It is possible, however, to exaggerate the importance of this fact. No organism reaches a high degree of complexity without adopting cellular structure. Some of the Siphonales show a limited degree of resemblance to higher plants in external form, and there is thus a suggestion of a much greater degree of differentiation of parts than in fact exists. Pressed specimens, too, tend to look more like higher plants than do these lowly organisms in their natural form. It is doubtful whether *Caulerpa* and its allies are in fact the most highly differentiated syncytial organisms. Some of the Ciliophora appear more complex. This most aberrant group, however, is excluded from consideration in the present paper, as it will be discussed under Proposition VI. Reasons for regarding the ciliates and their allies as syncytia will be given there. (See also Baker, 1948, *b* and *c*.)

At a meeting of a scientific society in Würzburg on 23 November 1878 Sachs demonstrated a series of Siphonales and remarked that these, as well as the Mucorineae, had up till then been regarded as 'einzellige'; he considered that they should rather be called 'nicht cellulare' (Sachs, 1879). It is to be noted that this demonstration took place seven days before Schmitz began to make known his discoveries on syncytial plants. Like everyone else, Sachs considered that the plants he called non-cellular were devoid of nuclei. When their nuclei were discovered, the name stuck to them; and indeed it is not inappropriate. It is unfortunate that the name was also applied by some writers to certain uninucleate protists, which evidently correspond in their structure to single cells. This matter will be discussed with Proposition VI; I have already commented upon it elsewhere (Baker, 1948, *b* and *c*).

Sachs reverted to the subject of non-cellular plants many years later in two important papers (1892 and 1895). He considered that the accepted terminology of cytology was misleading and should be changed. For him, the cell was the cell-wall, or sometimes the cell-wall with the contents of the cell (1892, p. 62; see also p. 166 of the present paper). He felt that a new word was required. 'Under the name of an Energid', he wrote (1892, p. 57), 'I think to myself of a single cell-nucleus with the protoplasm controlled (beherrschten) by it.' He chose the word *energid* to indicate that the vital activities reside in the nucleus and cytoplasm; he did not intend 'energy' to be understood here in its physical sense (1895, p. 410). He regarded the energid as a morphological as well as a physiological unit. It might produce a cell-wall or other secreted objects, or it might not. In most cases each cell is inhabited by one energid, but the Siphonales were obviously peculiar in this respect. Sachs had changed his mind; he now called them one-celled plants, but remarked that the cell was produced by numerous energids. The

difference between such forms as the Siphonales on the one hand and cellular plants on the other, in Sachs's terminology, was that the neighbouring energids of the former were not sharply marked off from one another (1892, p. 62; 1895, p. 425).

Sachs's new word was never widely accepted. Biologists might perhaps do well to reconsider their tacit rejection of it. His expression 'beherrschten' has gained rather than lost in significance since his time. A difficulty is that in syncytia we have generally no means of delimiting the nuclear zones of control, which must be constantly shifting in cases where the cytoplasm is in motion. Again, it is difficult to be certain that a particular part of the cytoplasm is 'controlled' by only one nucleus. Occasionally, however, the zones of control announce very clearly that they exist. A good example is provided by the syncytial zoospore of *Vaucheria*, in which two flagella are related to each nucleus.

Polyploidy

Boveri (1905) called the nucleus of the spermatozoon or egg a *Hemikaryon*; the fusion-nucleus of a zygote an *Amphikaryon*; and a nucleus in which the number of chromosomes had doubled without nuclear division a *Diplokaryon*. Strasburger was using the words *diploid* and *haploid* in 1907 (pp. 490, 529) and *tetraploid*, *oktoploid*, and *polyploid* in 1910 (pp. 422, 444), but it seems doubtful whether we have any authoritative statement of their meanings. A quotation from a well-known and excellent textbook will exemplify the doubt. Its author writes: 'The lowest diploid number found in any organism is 2, which occurs in the Roundworm, *Ascaris megalocephala* var. *univalens* (this species also has a tetraploid variety, *bivalens* with 4 chromosomes in the diploid set).' Thus in one sentence we are told that four is both the diploid and the tetraploid number of chromosomes in the variety *bivalens*. Some authors use the expression diploid as synonymous with the somatic number in sexually produced organisms, and haploid as synonymous with the gametic number, whether or not there happen to be only two sets of different chromosomes in the cells called diploid and one in those called haploid. I shall not follow this usage, but shall employ the word haploid to refer to a single set of different chromosomes and diploid to refer to two such sets, and shall use triploid, polyploid, &c., in conformity with this system. (Thus a gamete-nucleus is characteristically haploid, but may be diploid.)

The haploid protoplast, or *haplocyte* as I shall call it, is a better example of a unit than a *diplocyte*, with its two sets, just as a box containing the playing-cards of a single suit is more perfectly unitary than one containing two suits or the tetraploid pack; but since the great majority of organisms arise, directly or indirectly, from the fusion of two haplocytes, the most usual morphological unit in both plants and animals is the diplocyte. The degree of duplicity exhibited by this cellular unit is an expression of one of the most fundamental facts of biology. The *Uebereinstimmung* postulated by Schwann

was to some extent upset by the discovery that there are typically both haplocytes and diplocytes in organisms; but much more serious from the point of view of the cell as a morphological unit is the fact that polyploid nuclei also exist. The history of this discovery must now be briefly traced.

Guignard (1884, p. 27) suggested that certain plant-cells may contain about twice the usual number of 'bâtonnets chromatiques', but he was dealing with a normally haploid structure, and the discovery of polyploidy must be ascribed to Boveri (1887). The latter showed that there are two varieties of *Ascaris megalocephala*, which he called Typus Carnoy and Typus van Beneden, after earlier students of the chromosomes of this nematode. He showed that in Typus Carnoy there are two chromatic elements (chromosomes) in the ripe egg, in Typus van Beneden only one. Thus the somatic cells of the former variety were tetraploid. This case is not quite so simple as it appeared to be, for, as is well known, the chromosomes break into fragments in cells other than those of the germ-track; and when they have done so, the number in Typus van Beneden appears not to be exactly half that in Typus Carnoy (Walton, 1924). It seems allowable, however, in the present state of knowledge, to regard Typus Carnoy as tetraploid before fragmentation.

Boveri later (1903, *a* and *b*, 1905) shook the eggs of the sea-urchin *Strongylocentrotus* immediately after fertilization and by this means suppressed the first cleavage, while the chromosomes divided; he thus obtained tetraploid larvae experimentally.

A discovery in plants similar to Boveri's in *Ascaris* was made by Rosenberg (1903), who showed that *Drosera rotundifolia* has 20 chromosomes in its somatic cells, while *D. longifolia* has 40. Strasburger (1905) counted the number of chromosomes in the pollen mother-cells of *Alchemilla arvensis* and *A. speciosa*, and found that the latter had twice as many as the former. These were the first indications of what is now known to be the very widespread occurrence of polyploidy among higher plants. A complication is introduced by certain species which seem to have become secondarily diploid, with double the usual diploid number of chromosomes, by differentiation of the four sets into two.

Difficulties in the sex-determining mechanism prevent most dioecious animals from doubling their chromosome numbers throughout their tissues, but there are indications that certain hermaphrodites are polyploid (see White, 1940). There is no particular barrier against chromosome replication in somatic cells, and it is not unusual for some of these to become polyploid. The classical example of mosaic polyploidy is provided by the honey-bee. Petrunkevitch found long ago (1901, pp. 587–8) that there are only 16 chromosomes in the first division of the nucleus of the drone-egg, but about 64 in cells of the blastoderm of the later embryo. In a celebrated paper Meves showed that while the diploid number (counted in the oogonia of the queen) is 32 and the haploid 16, more than 60 chromosomes are present in the follicle-cells of the testis (1907, pp. 471–2). Nowadays we have simpler

methods of detecting polyploidy, in particular cases, than laborious chromosome-counts. We may measure nuclear volumes (Jacobj, 1925), or count either the nucleoli (especially where there is one per haploid set in early prophase (de Moll, 1923, 1928)), or the heterochromatic X-chromosomes (Geitler, 1937), or heterochromatic satellites (Berger, 1941). The most extreme instance of mosaic polyploidy seems to be provided by the pond-skater, *Gerris lateralis*, in which the degree of replication reaches 1024-ploidy, or even farther, in the salivary glands (Geitter, 1938).

There is evident similarity between multinucleate conditions and polyploidy. There is general correspondence between a single mass of cytoplasm containing two diploid nuclei and another containing one tetrapoid nucleus: both may be called tetraplocytes. Particular tissues tend to provide examples of both the binucleate and the uninucleate tetraploid states. Thus in the roots of *Pisum sativum* treated in life with chloral hydrate, Strasburger (1907, pp. 484 5) found both mitoses with tetraploid chromosome-numbers and binucleate cells. In the tapetal layer of the anthers of certain plants, some cells show polyploid chromosome-numbers at division, while others are bi- or multinucleate (see especially Witkus's study of *Spinacia* (1945)). In certain parts of young seedlings of *Allium cepa*, again, a number of tetraploid cells are formed in certain regions; in the same sites binucleate cells are common (Berger and Witkus, 1946). In the mammalian liver there are polyploid cells of various degrees of chromosome-replication; there are also bi- and multinucleate cells (Wilson and Leduc, 1948). Fell and Hughes (1949, p. 366) have shown by the study of living tissue-culture cells of the mouse that polyploid nuclei may arise by mitosis of binucleate cells, a single spindle being formed for the chromosomes of the two nuclei; fusion of diploid nuclei and endomitosis are other mechanisms by which the same end is achieved (Berger, 1937; Geitler, 1939; Wilson and Leduc, 1948).

The 'polyenergid' nuclei of certain Protozoa will be mentioned later under the heading of Proposition VI. I have already discussed them elsewhere (Baker, 1948*b*).

The existence of polyploidy undoubtedly constitutes an exception to the general rule of the 'Uebereinstimmung' of all cells. One polyploid cell cannot be regarded as homologous with one diploid cell. Boveri (1903*a*), for instance, found that the protoplasts of tetraploid larvae of *Strongylocentrotus* were much larger and fewer than those of the diploid form; in the case of the mesenchyme he found that there was about half the normal number. Thus one tetraploid corresponds with two diploid protoplasts. Polyploidy, however, is clearly a secondary condition. Diplocytes and haplocytes are the characteristic primitive morphological units of plants and animals, and are still retained as the elementary components of most organisms. Those organisms that show mosaic polyploidy have haploid germ-cells and are everywhere diploid in the early embryonic stage of the sexually produced form.

The Indivisibility of Cells into Smaller Homologous Units

Hirsch remarks (1942) that the body of an organism consists of a series of 'partial systems', each of which (till protons and electrons are reached) is built of partial systems of a lower order. Thus the body is made up of organs which are divisible into tissue-units and these into cells; the latter contain *Mikronen* (mitochondria, Golgi-bodies (lipochondria), various granules and vacuoles, muscle-fibrils, chromosomes, nucleoli), and these are composed of sub-microscopic *Submikronen*, themselves made up of large molecules; and so on.

If an object is composed of parts, all of which are divisible into smaller parts that show what Schwann called 'Uebereinstimmung' with one another, then these smaller parts are clearly the true units of construction. It is important to stress the fact that the Mikronen are not homologous parts: a muscle-fibril, for instance, does not correspond, in any predicable way, to a nucleolus. Further, the Mikronen taken together do not constitute the cell, which consists largely of ground-cytoplasm and nuclear sap. There is no intention here to criticize Hirsch's analysis adversely, but only to point out that it in no way invalidates the cell-theory. *The cell is not composed of any lesser homologous units*, other than those minute particles that compose all matter, and to these the idea of homology does not properly apply. As Hanstein remarked long ago: 'In the last resort the *protoplast*, *not* the *molecule* or the *micelle*, is the organic individual' (1880, p. 295). With the reservations that have already been noted, this is true.

Comment

Adequate critiques have already been given in this paper of the various facets of the cell-theory that are included under the head of the second part of the second Proposition. It remains to make one general comment. Whenever a student wants to 'understand' a complex histological object that is unfamiliar to him (a Pacinian corpuscle will serve as an example) or a research-worker to grasp the minute structure of a previously unknown organ, he proceeds first of all to try to determine where the boundaries of the protoplasts are—what is cellular and what is intercellular. In other words, he tries to interpret what he sees in terms of the part of the cell-theory that is summarized in Proposition II, in the formulation here adopted. That is the measure of the homage he pays (often unwittingly) to the founders of the cell-theory.

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The History of the Cytoplasmic Elements during Vitellogenesis in *Drosophila melanogaster*

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Summary

1. At least two kinds of yolk are developed in the eggs of *Drosophila melanogaster*. The origin of the proteid yolk has been traced to precursors sent into the oocyte by the nurse cells, while the minute fatty yolk granules are transformed chondriomites also contributed by the nurses. The behaviour and morphology of Golgi material in the nurses indicate that it may be responsible for a third type of yolk.

2. A yolk nucleus has been found in oocytes of an age when the germinal disk has been pushed to one side. So long as such a structure exists, all the proteid yolk precursors are concentrated and matured within its confine. Soon this structure disintegrates and disappears. But after its disappearance, yolk precursors continue to be sent by the nurses into the oocyte, and there they develop into mature granules apparently without the benefit of a yolk nucleus. On the basis of facts thus far obtained, it is, therefore, difficult to say just what role the so-called yolk nucleus plays in vitellogenesis in *Drosophila melanogaster*.

3. Golgi material in the nurse cells has been observed to undergo two distinct proliferations. In young nurses, Golgi material occurs in a few discrete bits which, as the nurses grow in size, proliferate into a large number of intertwining threads so arranged as to suggest a net. Then follows a period in which only very few minute granules of Golgi material can be seen. This is definitely a period of scarcity. After this, a second proliferation takes place. This one is carried to such an extreme extent that the cytoplasm of the cells is just black with Golgi material. The Golgi threads, arranged more or less in a parallel fashion and very closely together, present the appearance of pieces of Golgi 'tissue'. Next, the pieces of 'tissue' loosen up into separate threads from which individual Golgi granules arise. These granules enlarge to become vesicles.

4. In the course of development of the nurse cells, the mitochondria also undergo a proliferation. Finally, the chondrioconts break up into granules which enlarge and eventually all transform into droplets of unsaturated fat.

5. My observations point to the conclusion that the oocyte depends upon its nurses for the production of fatty yolk. Proteid yolk granules, however, are developed within the oocyte, though from precursors sent in also by the nurse cells. The follicular cells do not appear to contribute anything to vitellogenesis in *Drosophila*.

6. An idiosome-like granule has been observed in oogonia, and also in oocytes up to a certain stage of development. For reasons stated elsewhere, it cannot be regarded as an idiosome nor as a centrosome. This structure has not been observed in nurse cells.

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Introduction

THIS paper aims to report some observations made on Golgi material and mitochondria in relation to yolk formation in *Drosophila melanogaster*. I have limited this study to the period of development beginning with the oogonial stage through the differentiation of oocytes and nurses to the stage of development of the egg when all its nurse cells have been absorbed.

For a description of the organization of the ovaries of *Drosophila*, reference may be made to Guyénot and Naville (1933), Miller (1950), and Yao (1949). For the condition of the chromosomes in the oocyte and the nurses during the period of development covered in this study, readers are referred to Guyénot and Naville (1933), and Painter and Reindorp (1939) respectively.

Material and Methods

The flies used were from 'wild' stocks maintained in our genetics laboratory. Culture conditions were the same as I have reported in connexion with a previous study (1947), except that for the present study flies were raised under a controlled temperature of 22° C.

Depending upon the stages of development needed, ovaries were dissected out from pupae or from flies hatched within 24 hours from female pupae previously segregated from male ones and left in fresh bottles of food. Dissections were done in a drop of physiological saline, and the ovaries were then immediately immersed in the fixatives.

For demonstrating Golgi material, I have tried such silver methods as Aoyama and Da Fano. In spite of numerous variations in the duration of fixation, impregnation, &c., I have obtained only very poor results with them. Warm osmic methods, such as Kolatchev's, have yielded less discouraging slides. But osmic impregnation at room temperature, according to Mann-Kopsch, for instance, has proved in this, as in my other studies, to be the most satisfactory.

For mitochondria, my experience with the various standard fixing and staining procedures has inclined me to rely on Champy's fixative followed by Benda's crystal violet method as modified by Baker. This combination has not only the virtue of giving a robust demonstration of mitochondria but also that of demonstrating the chromosomes in a very desirable state.

Observations

Mitochondria. The oogonia when not in mitotic division show the mitochondria, mostly granules or short rods, in an uneven perinuclear distribution (fig. 1A). But when mitosis has set in, the mitochondria are distributed evenly in the cytoplasm (fig. 1B). Fig. 1C shows the disposition of mitochondria in an oogonium at mitotic metaphase.

At a stage when an oocyte is differentiated from its nurses, the mitochondria are generally found in some such condition as shown in fig. 1D. In both the

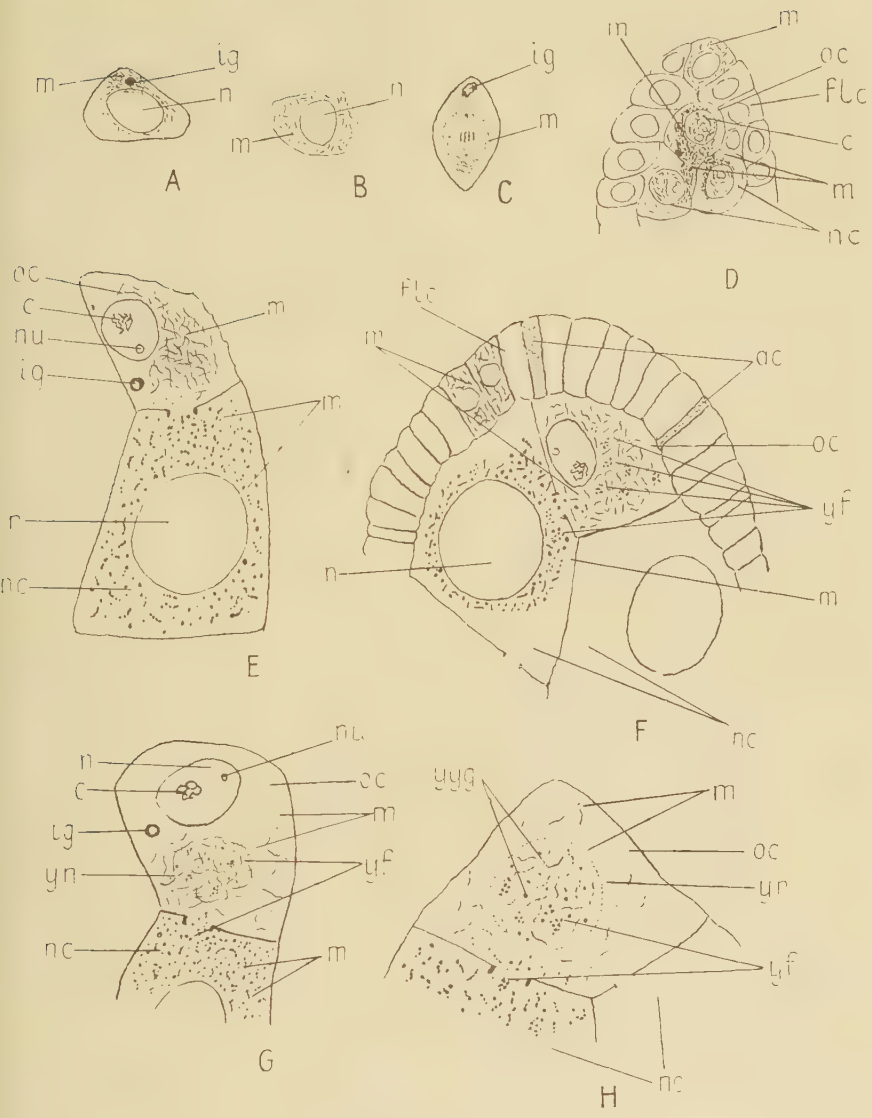

FIG. 1. Champy-Benda preparations, × 1,300.

A–C oogonia; D, tip of a cyst; E, an oocyte and a nurse; F, tip of a cyst; G, an oocyte and a portion of a nurse; H, an oocyte and portions of two nurses.

These symbols are used in this and subsequent figures:

c, chromosomes; *dc*, dark cell; *fl*, follicle; *flc*, follicle cell; *fy*, fatty yolk; *fyv*, fatty yolk vacuoles; *g*, Golgi material; *ig*, idiosome-like granule; *m*, mitochondria; *n*, nucleus; *nc*, nurse cell; *nu*, nucleolus; *oc*, oocyte; *og*, oogonium; *py*, proteid yolk; *v*, vacuole; *yf*, yolk flake; *yn*, yolk nucleus; *ypr*, yolk precursor; *yyg*, young proteid yolk granule.

oocyte and the nurse cells, the mitochondria seem to show a definite tendency to concentrate around or to one side of the nucleus.

At the next stage, the chondrioconts in the nurses show an increase in thickness and a breaking-up into chondriomites. But those in the oocyte preserve their thread form, and, apparently, retain their original diameter. In fact, they appear to be longer than in earlier stages (fig. 1E). From the stage indicated in fig. 1E onward, the mitochondria in the oocyte always stain less robustly than those in the nurse cells. Although the nurses almost constantly send into the oocyte their stock of mitochondria, it seems that the mitochondria from the nurse cells do not need to have travelled very far into the oocyte before they lose a part of their power for retaining the stain.

Fig. 1F shows that in the cytoplasm of the oocyte and the attached nurse there are present a number of small flakes of irregular shapes which stain less intensely than the mitochondria but in which are embedded minute granules which stain just as intensely as the mitochondria. These flakes, which henceforth will be referred to as the proteid-yolk flakes while the minute and almost perfectly spherical and very intensely staining granules embedded in them will be known as the proteid-yolk precursors, are not indigenous to the oocyte. Their origin has been convincingly observed to be in the nurse cells (figs. 1, F–H; 2A).

As the oocyte grows to the stage shown in fig 1E, its nucleus is placed to one side of the cell and its mitochondria tend to congregate into a zone within which are caught practically all of the yolk flakes visible in the oocyte (fig. 1, F and G). The area marked out by the concentration of mitochondria in an oocyte such as shown in fig. 1G appears darker than the general cytoplasm. Careful focusing has convinced me that the difference in colour between this area and the general cytoplasm is not due to the concentration of mitochondria but is due to the existence of a different substance. Fig. 1H shows the area with the embedded mitochondria, yolk flakes, and free young yolk granules; while fig. 2A shows the yolk flakes and young yolk granules without the mitochondria. When completely formed, this area has been invariably found to be in reality an almost perfect sphere with a very smooth surface. I propose to call this structure a yolk nucleus.

In Champy material stained in crystal violet, the more mature yolk granules always appear yellow with a thicker or thinner purple rim depending upon the size of the granules. Before the yellow centre is visible in them, the apparently homogeneous young yolk granules stain about the same shade of intense purple as the mitochondria, unless the degree of differentiation has been exactly right. Since some mitochondria may appear in the form of dots about the same size as the young and homogeneous yolk granules, it is therefore difficult, though not really impossible, to differentiate them. Fortunately, however, if the Champy slides are bleached in turpentine for about an hour before stain is applied, the property of retaining the purple colour on the part of mitochondria in the oocyte is greatly reduced and they invariably appear in a rose colour. The young yolk granules, on the contrary, stand out most

strikingly in an intense purple colour in such preparations. The minute dots seen embedded in yolk flakes also retain this same shade of intense purple. It was the sight of preparations like the one represented by fig. 2A that con-

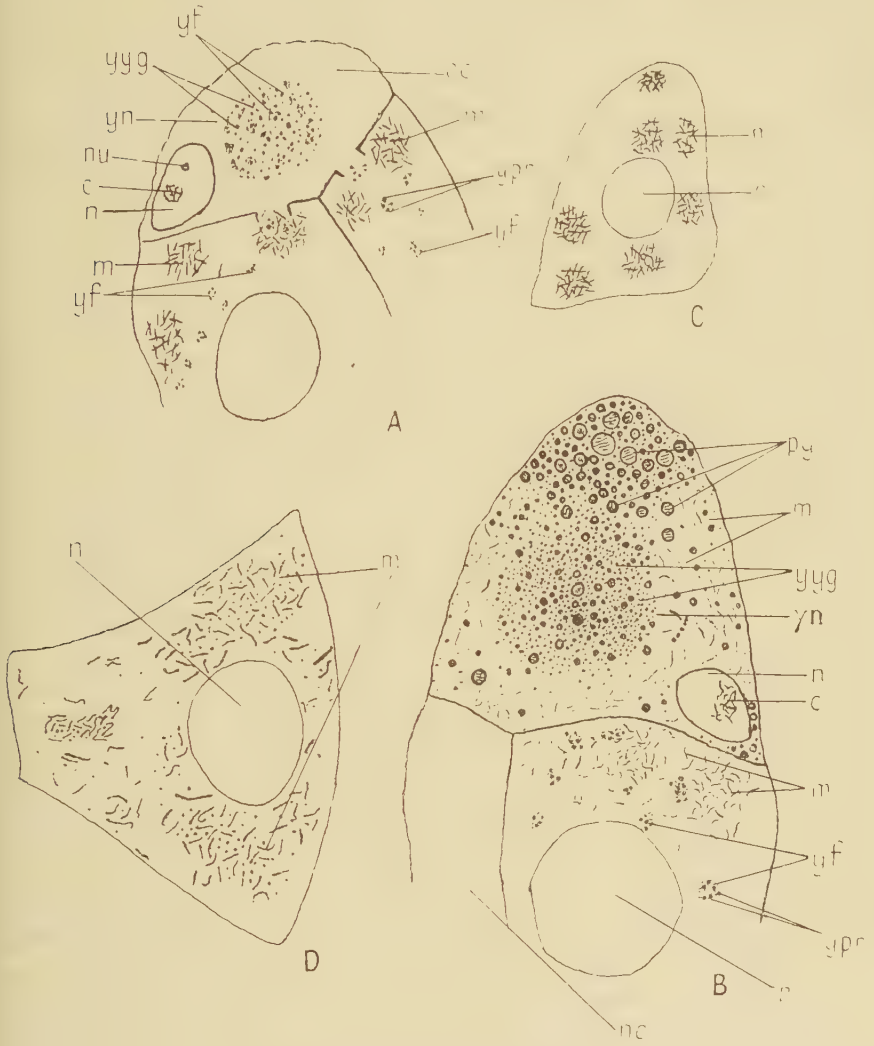

FIG. 2. Champy-Benda preparations, × 1,300.
A–B, oocytes and portions of nurses; C–D, nurse cells.

vinced me that the minute dots embedded in the yolk flakes are the original substance from which one type of yolk in *Drosophila* eggs developed. They are the precursors to this type of yolk. Since this type of yolk persists in material preserved in acid fixatives such as Bouin, mercuric acetic, &c., I regard it as of a proteid nature.

As far as microscopic observations can ascertain, the individual yolk precursors are released as a result perhaps of the dissolution of the yolk flake matrix. I consider the minute granules embedded in the flakes as proteid-yolk precursors on the following evidence: (1) They show the same stain reaction as the free homogeneous-staining young yolk granules which are distinguishable, on the basis of colour reaction, from chondriomites; (2) The smallest homogeneous young yolk granules often occur in groups very suggestive of the arrangements in which yolk precursors are embedded in the yolk flakes.

Once the yolk flakes are inside the oocyte they apparently all move into the yolk nucleus, if such a structure is already formed and is still existing. But apparently the yolk nucleus is not a necessary factor contributing to the change of the yolk-precursors into proteid-yolk granules, because the supplying of yolk precursors to the oocyte by the nurses persists long after the yolk nucleus as such has disappeared in the oocyte (fig. 3A). The disintegration of the yolk nucleus is illustrated in fig. 2B. The breaking away of the yolk granules from the yolk nucleus seems to be the first visible sign of this disintegration, leading to the complete disappearance of this structure. After that, proteid-yolk granules of all sizes together with yolk flakes are seen rather uniformly distributed among the mitochondria in the ooplasm, as shown in fig. 3A.

Regarding the mitochondria in the oocyte and its nurses, from the time when the yolk nucleus begins to take shape (fig. 1E) up to the time represented in fig. 2A, the situation is as follows: in the oocyte, the mitochondria remain as comparatively long threads, and most (though not all) of them are taken into the yolk nucleus (fig. 1, G and H). In the nurses, however, the mitochondria tend to become fatter and to break up into granules or short rods whose granular nature is unmistakable. Besides these changes they also show a tendency to form clumps. At the height of this process there are hardly any mitochondria to be seen outside of the few clumps (fig. 2, A and C).

This clumping is but a temporary situation and one of rather short duration. A very interesting thing about such cells in which clumping is seen is that their cytoplasm appears microscopically structureless. Areas in the cytoplasm not occupied by clumps of mitochondria or structures such as yolk flakes appear empty. One is left with the impression after viewing many nurse cells at this stage that some kind of structural reorganization has taken place in the cytoplasm and that the mitochondria have been passively lumped together rather than having come together as a result of some changes inherent in themselves. This impression is strengthened by the fact that at the same stage of development the Golgi material in the nurse cells also undergoes a period of clumping (fig. 5B). When the mitochondria in the nurses are released from clumping, they appear more swollen, and more of them are in the form of granules or short and loose strings of beads (fig. 2D).

In nurses which have attained their maximum size, the mitochondria are practically all in granular form. This almost complete granulation on the part of the chondriome is but the culmination of a process that has begun in very early stages. What is interesting about them now is that they have acquired

uch a fatty constitution that they appear black in unstained Champy material. n Champy-Benda slides, no purple mitochondria can be seen in nurse cells f this age. Slides stained in Benda's crystal violet after a period of bleaching n turpentine, however, do show granular mitochondria in an extremely faint

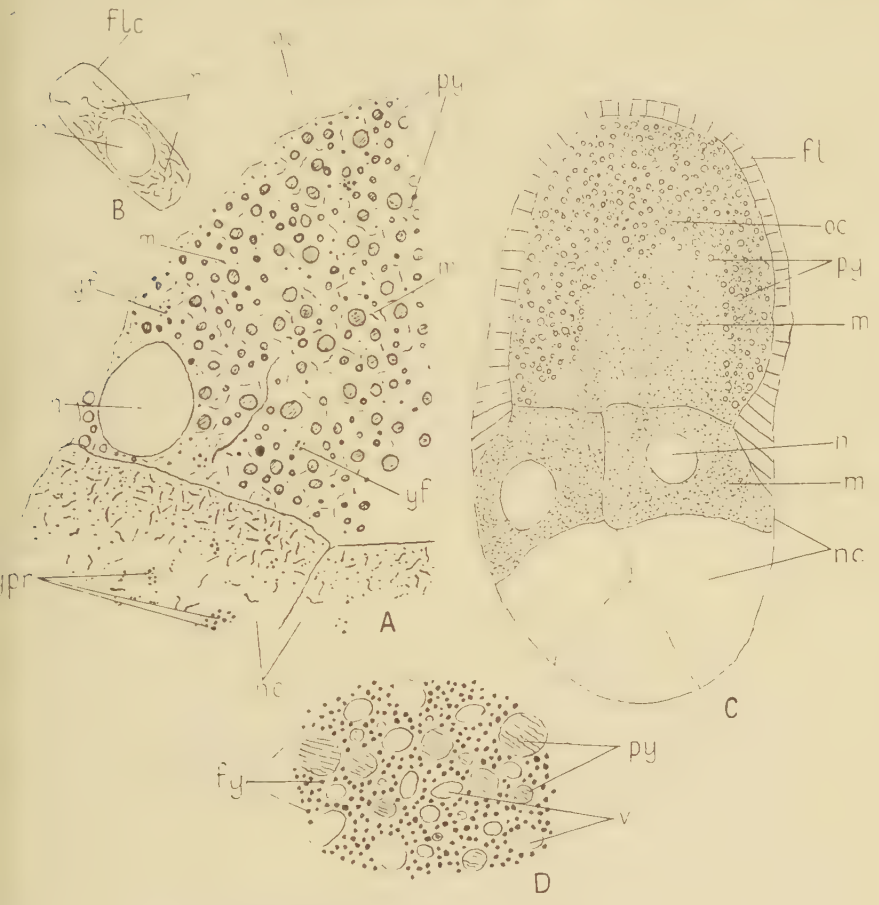

FIG. 3. Champy-Benda preparations, × 1,300.
A, portion of an oocyte and a nurse; B, a follicle cell; C, a section of a cyst; D, a portion of an oocyte.

ddish colour. It seems, therefore, that the mitochondria at this stage has ndergone some kind of chemical change. In this change its unsaturated fatty ntent is increased while its power of taking the ordinary mitochondrial ain is much reduced. And, in this changed state, they are sent into the oocyte large quantity (fig. 3C). It seems that the change does not stop at this stage, ut proceeds to completion when the partially changed mitochondria granules e within the oocyte, as will be discussed presently.

The appearance of rather large vacuoles of various sizes in the ooplasm is sign that the egg has become more mature than the oldest stage I have so

far described. If an egg of this maturity is stained in Benda's crystal violet after bleaching in turpentine, not the faintest shade of colour could be imparted to the transformed chondriomites. They all appear as a second category

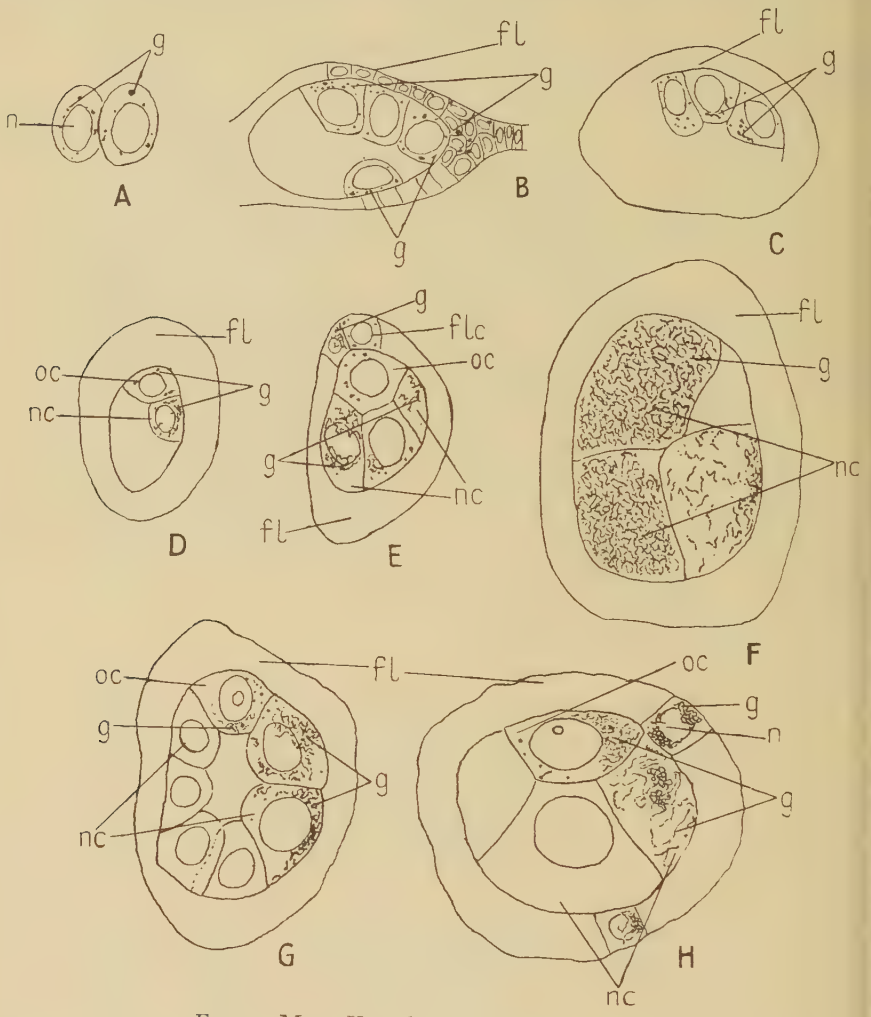

FIG. 4. Mann-Kopsch preparations, × 1,300.
A, oogonia; B–H, sections of young cysts.

of vacuoles, much smaller than the large ones just mentioned and of very uniform size. They impart to the ooplasm a spongy appearance. I take this to mean that the original mitochondrial material has been entirely transformed into unsaturated fat. In unbleached Champy-Benda slides, however, a piece from an oocyte of the same maturity would show: (1) large proteid-yolk granules of all sizes, grading down to the size of the yolk precursors; (2) large vacuoles; (3) numerous minute black granules which are the transformed

granular mitochondria (fig. 3D). I regard them as the fatty yolk in *Drosophila* eggs.

Golgi material. The Golgi material in the oogonia is in discrete bits whose sizes vary somewhat (fig. 4A). Fig. 4B illustrates the condition of the Golgi material in a few cells in a cyst, among which it is yet difficult to distinguish the oocyte from its nurses. Fig. 4C shows three cells at a rather later stage of development. In two of the three cells the Golgi granules have begun to line into threads. This may be looked upon as the beginning of the first of two proliferations on the part of Golgi material in the nurse cells. In fig. 4D, the Golgi material in one of the two cells is on the way to develop into a net of some kind. The Golgi material in the other cell, however, still appears as individual bits. On the basis of the condition of its Golgi material, this cell may be judged as an oocyte since it is a fact ascertained in my material that cytoplasmic changes start earlier in nurse cells than in the oocyte which they supply. Fig. 4E shows an easily identifiable oocyte and its nurses. The Golgi material of the oocyte is still in discrete bits with but little increase in quantity. In its nurses, however, especially in one of them, the situation, so far as it concerns the Golgi material, has already become very obvious—it is unmistakably developing into a net of very tortuous threads around the nucleus, eventually, in more mature cells, spreading throughout the cytoplasm (fig. 4F).

In the stage represented by fig. 4G, proliferation of the Golgi material into a sort of net has also started in the oocyte. That the Golgi net in the oocyte will eventually develop as extensively as it does in the nurses is shown in figs. 4H and 5A. For the sake of accuracy, it is necessary to point out that the form of net whether in an oocyte or in a nurse is perhaps rather apparent than real, because by careful focusing one feels convinced that the net appearance is due to a large number of interweaving and criss-crossing fine threads. In addition, it should also be pointed out that the threads suggest alined minute granules. Individually, the granules are almost at the border of microscopical vision.

So far as this first proliferation of Golgi material in the nurses is concerned, the condition shown in fig. 4F is the climax. Further development would bring the Golgi material in the nurses to the state depicted in fig. 5B. It is difficult to speak with confidence, but it seems reasonable to believe, after careful study of many slides, that something has happened within the cytoplasm, as has been mentioned in connexion with mitochondria, which causes the Golgi material to undergo a very tight clumping. The threads appear in separate, entangled masses.

Fig. 5C shows that the Golgi material in the nurse cell has been released from clumping. This stage is followed by a period during which most of the Golgi material in the nurses seems to have either actually disappeared or have lost the power to reduce osmium tetroxide. At any rate, there appear in the nurses only relatively few minute individual granules or groups of them, some of which are arranged into short strings of loosely connected beads (fig. 5, D

and E). In extreme cases, Golgi bodies are hardly to be seen in nurses of this stage of development, while in cells of stages younger or older than this on the same slide the Golgi material is abundantly demonstrated.

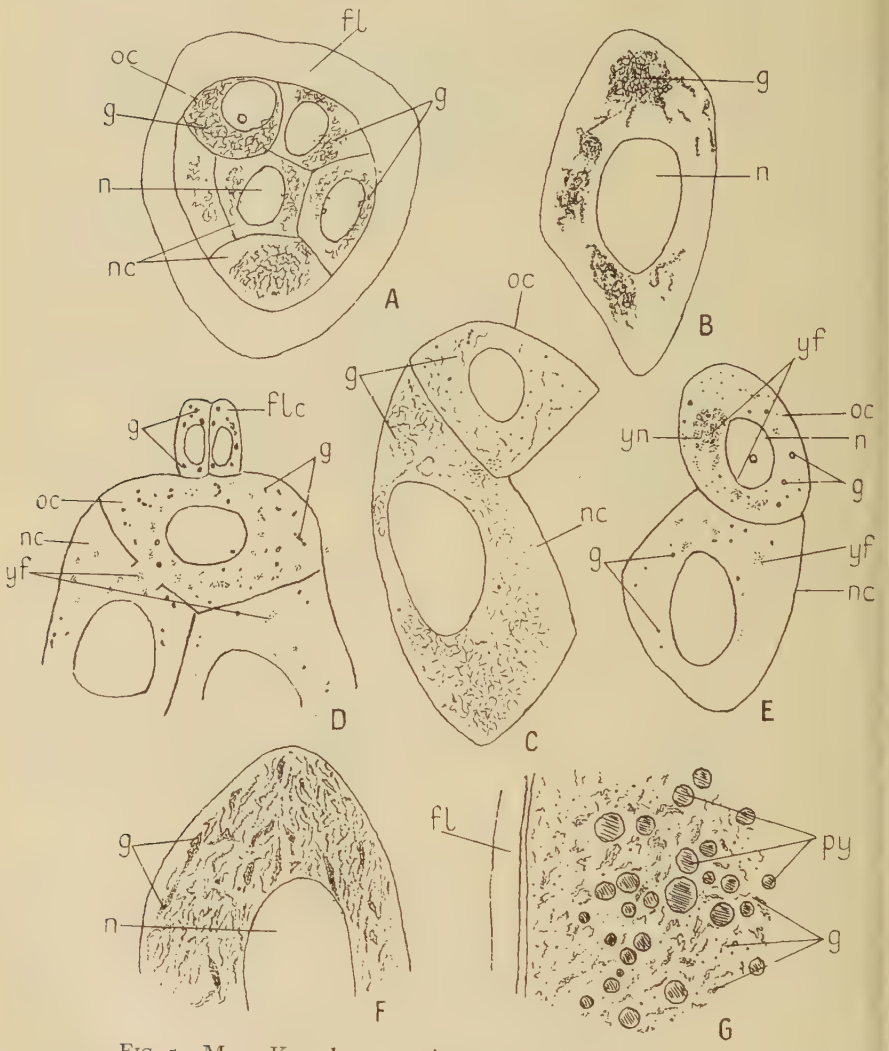

FIG. 5. Mann-Kopsch preparations, × 1,300 (except C, × 300).

A, section of a cyst; B, a nurse cell; C, an oocyte and a nurse; D, an oocyte, two follicle cells, and portions of two nurses; E, an oocyte and nurse; F, portion of a nurse; G, portion of an oocyte.

The same two figures also illustrate the condition of Golgi material as found in the oocyte in the stage of development represented. Here, the Golgi net developed in earlier stages has broken up. Morphologically, the Golgi bodies in the oocyte now are about the same as those in the accompanying nurses,

though some of them may show a lighter centre, especially if the sections have been bleached for at least half an hour in turpentine. Quantitatively, however, they are more abundant as compared with what is visible in the nurses. The single or aggregated Golgi particles are distributed evenly through the ooplasm.

It will be noticed in fig. 5D that yolk flakes are abundantly present but diffusely distributed in the oocyte. Fig 5E shows most of the yolk flakes taken into the yolk nucleus, and those lying in the general cytoplasm will presumably be taken in to enlarge it. I have never seen any concentration of Golgi material in the yolk nucleus.

The next change undergone by the Golgi material in the nurses is apparently a sudden one, because I have seen hardly any transitional stages. This change consists in a second proliferation which is much more intense than the first one observed in the earlier stages of development as illustrated in fig. 4F. As a result of this proliferation, the cytoplasm is just black with Golgi material. Fundamentally, it exists in the form of fine and tortuous threads, the granular nature of which I have already mentioned above. But instead of giving a net appearance, the fine threads now often lie more or less parallel to one another and so closely together that, partly owing to their twisted condition, they form large pieces of 'Golgi tissue' in the cytoplasm (fig. 5F).

Further change in the Golgi material is shown in fig. 6A in which the nurse cell reveals only part of its Golgi material content still concentrated in a state suggesting pieces of fabric; but short tortuous threads and unconnected individual granules are seen distributed throughout the cytoplasm. So in nurses of this age, there occurs in the Golgi material a second period of granulation.

As to the condition of Golgi material in the oocyte from the stage as shown in fig. 6A on to a time when there are yet 2–4 nurses not absorbed but somewhat reduced in size, it may be said that it largely reflects the condition of this cytoplasmic element as seen in the attached nurses (fig. 6, A–C). Fig. 6B represents a portion of the nurse which supplies the oocyte of which fig. 6C depicts a small section. Most of the Golgi material in these cells are separate particles, much larger than those still alined into threads. Each particle shows a light centre, appearing in optical section as a complete or broken ring.

The next advanced stage would be an oocyte having absorbed all its nurses. In such an egg, practically all its Golgi material content is in the form of complete or broken rings (fig. 6D). At this stage in the ooplasm there are, besides the big vacuoles, also minute 'vacuoles' which are so numerous as to impart to the ooplasm a spongy appearance. These 'vacuoles' are really the transformed granular mitochondria which are not stained in Mann-Kopsch material.

Golgi material and mitochondria in the follicle cells. Apparently the principal role in vitellogenesis in *Drosophila melanogaster* is played by the nurse cells. As far as microscope can reveal, the follicle cells do not seem to contribute anything to the process. I have often seen so-called 'dark cells' like those described by Peacock and Gresson in the sawflies (1928). In such cells it is

impossible to distinguish any cytoplasmic or nuclear structure. I have observed no transference of material from these or other cells of the follicular layer to the oocyte or its nurses (fig. 1F).

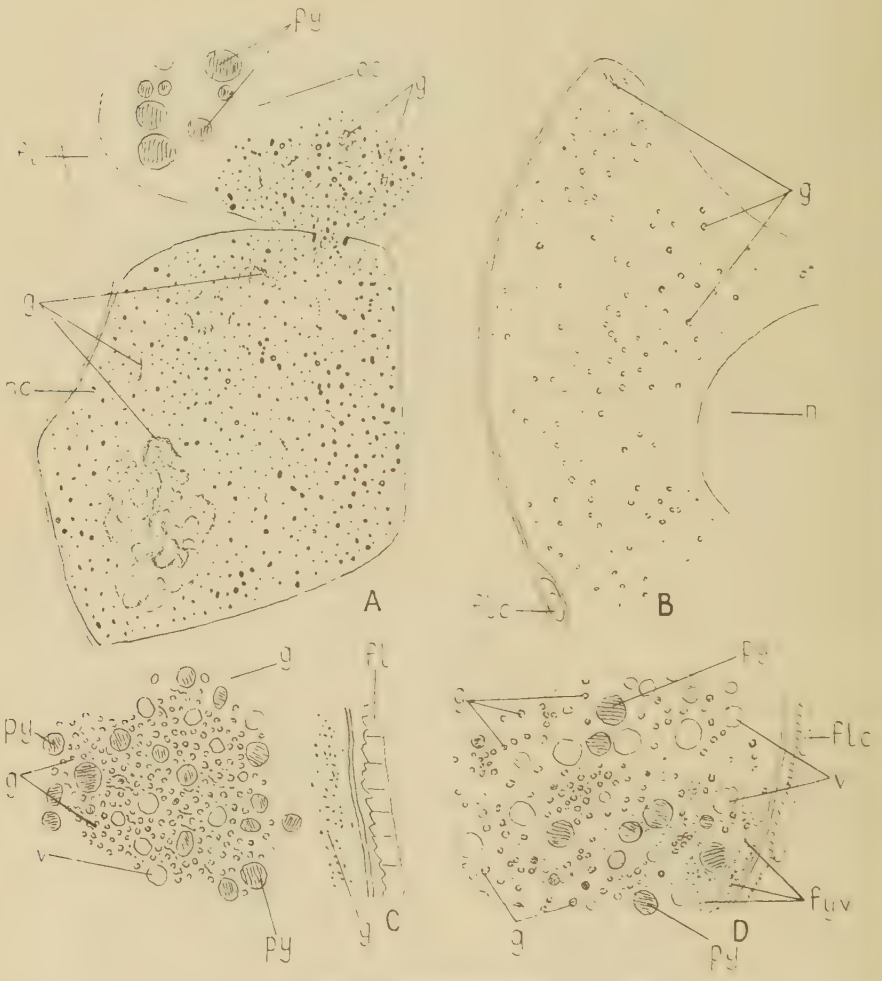

FIG. 6. Mann-Kopsch preparations, × 1,300.

A, portion of an oocyte and a nurse; B, portion of a nurse; C–D, portions of two different oocytes.

The changes seen in the Golgi material and mitochondria in the follicle cells of earlier stages of development seem to echo generally those undergone by the two cytoplasmic elements of the oocyte and its nurses. This is illustrated for the mitochondria in figs. 1, D and F; 3, A and B. In the older stages, when follicle cells are much reduced in height, it becomes increasingly difficult to demonstrate mitochondria in them.

Golgi material in the follicle cells of early stages also goes through a series of changes in morphology, generally echoing what is happening in the oocyte and nurses. According to the stage of development, they may appear as granules (fig. 4B), tortuous threads (fig. 4E), net (fig. 4H), and again granules (fig. 5D). In older follicle cells when they have become flat, nothing which can be confidently regarded as Golgi bodies has been seen. Since the follicle cells at such stages are in reality going through a sort of degeneration, their Golgi material and mitochondria must have suffered chemical changes so as to make it impossible to demonstrate them with the standard methods.

An Idiosome-like granule. I have observed this granule in oogonia and also in oocytes up to a certain size. This is a very puzzling structure, so far as I have studied it. When the mitochondria are congregated into a cap over the nucleus in an oogonium, this granule is seen apparently embedded in the mitochondria mass (fig. 1A). In Champy-Benda or Mann-Kopsch-Altmann slides it is observed to consist of a black or intensely-staining rim around a lighter centre. Its apparent structure and its positional relation to the mitochondria, though a granule suggesting a centriole has not been observed within the central light material, would remind one of the idiosome found in the auxocytes. In that case, the darker rim could be interpreted to be Golgi material or, at least, the osmiophilic part of Golgi bodies. But in view of the demonstrated distribution and morphology of Golgi material in the oogonia (fig. 4A), it seems odd that a portion of these cytoplasmic elements should assume a second type of morphology and distribution in the cell. At any rate, its being found in oogonia makes it difficult to regard this structure as an idiosome.

It is also difficult to regard it simply as an undivided centrosome, since, as far as I could ascertain, it is always single and without any connexion with the centrioles in dividing oogonia. In dividing oogonia, it shows signs of disintegration: its smooth outline is lost and the rind appears to have broken into separate short rods, giving to the whole structure a net-like appearance (fig. 1C). In young oocytes it does not show any tendency to stay near the nucleus, and its contact with the mitochondria, such as is observed in oogonia, is also lost. Neither have I ever observed it to be within the yolk nucleus (fig. 1G). All these may be considered as points unfavourable to interpreting this structure as idiosome. One interesting thing about it, however, is that in the oocyte, before it shows signs of disintegration, which occurs soon after vitellogenesis has set in, it is always to be found near that end of the oocyte which is next to the nurses. Later, when its disintegration has set in, this position is no longer invariable.

It may be mentioned here that what I have written about this granule agrees rather well with the description given by Harvey (1927) of the so-called yolk nucleus in *Ciona intestinalis*. But owing to the presence of another structure in my material better qualified to be called a yolk nucleus, and the fact that both Harvey's yolk nucleus and this granule have not been observed to have anything to do with yolk formation, I prefer not to describe this granule as a yolk nucleus.

It is difficult to say whether this granule is present in cells which are yet to be differentiated into oocyte and its nurses; for such cells are extremely small. But I am certain that I have not been able to observe it in nurses when they have become sufficiently large to make identification easy.

Discussion

So far as the proteid yolk granules found in the oocyte of *Drosophila melanogaster* are concerned, my observations have convinced me that their origin lies neither in the Golgi material nor in the mitochondria, both of these two types of cytoplasmic element having been demonstrated in my material. Also, the distribution and behaviour of the Golgi material and the mitochondria in the oocytes and their nurses throughout the period of development covered in this study do not reveal any visible connexion between them and the growth and maturing of the proteid yolk granules. The origin of this type of yolk has been traced to minute spherical yolk precursors embedded in flakes sent into the oocyte by the nurse cells from time to time. As to whether these flakes have a nuclear origin or arise independently in the ground cytoplasm, I am as yet unable to say. Nucleolar emissions have not been observed in these cells. Neither have successful preparations aiming to demonstrate desoxyribonucleic and ribonucleic acids proved of much help in this regard.

The so-called yolk nucleus as found in the *Drosophila* oocyte is a simple one. It certainly is not so complicated a structure as that described by Munson (1912) or Koch (1928) in spiders. Often a vague fibrillar structure may be detected in some yolk nuclei in my material; but that appearance is due to the presence in them of relatively long chondrioconts. According to Munson, the yolk nucleus in the spiders originates from a typical centrosphere of the oogonium. In *Drosophila*, however, even if the granule which I have seen to exist in oogonia and to persist in oocytes of an age when a yolk nucleus is already formed were a centrosome, its position and disintegration, all observed to be outside of the yolk nucleus, do not point to any morphological connexion between the two structures (fig. 16).

In the insect *Libellula depressa*, Hogben (1921) found a crescentic structure which he regarded as yolk nucleus. He further stated, 'It appears that the yolk nucleus is in reality the mitosome or chondriome of the oocyte, being the area in which the mitochondria are congregated.' According to Hogben, then, the yolk nucleus in *Libellula* is only an area in the cytoplasm wherein the chondriome of the oocyte congregate: apparently no specially differentiated cytoplasm is involved in this area. My observations on the *Drosophila* oocyte agree with this interpretation very well with regard to the mitochondria. But I have reason to believe that there is really a differentiated cytoplasm forming a sort of background material in which the mitochondria and yolk flakes are concentrated. True, this background material as found in the *Drosophila* oocyte could be interpreted to be the dissolution product of the matrix of the yolk flakes, thus denying that the yolk nucleus is composed of a mass of differentiated ooplasm. If it is the liquified matrix of the yolk flakes that gives

ise to the background material, then the yolk flakes found scattered in the general ooplasm in more mature oocytes in which no yolk nucleus is any onger visible should offer good opportunities for an observer to see coloured nasses of liquefied matrix. But no such coloured, disorganized mass has been een. I have noticed only intact yolk flakes and separate minute yolk granules o grouped together as to suggest the grouping of the yolk precursors em- bedded in the flakes. This would seem to indicate that the matrix of the yolk lakes loses its ability to take stain before it disintegrates to release the em- bedded yolk precursors. So, the coloured background material in the yolk nucleus could not be regarded as having originated from the liquefied matrix of the yolk flakes.

It is also difficult to say just what part such a yolk nucleus as I have found n *Drosophila* plays in vitellogenesis. Precursors of proteid yolk are transported nto it from the nurses. All the yolk granules are seen to increase in size and nost of them to acquire a yellow centre (in Champy-Benda material) while confined within the yolk nucleus as long as the latter persists. This may be aken to mean that a yolk nucleus plays at least the role of maturing proteid olk granules. But this is controverted by the fact that long after the yolk nucleus has disappeared, yolk flakes are still constantly sent into the oocyte nd their loads of precursors released and matured into the large proteid olk granules in the general ooplasm without the benefit of a yolk nucleus. urther investigation is required to settle the nature of the object that I have alled a 'yolk nucleus' for lack of a better name.

In *Drosophila melanogaster* I believe I have good evidence to say that the numerous and minute fatty yolk granules seen in oocytes when all its nurses have been absorbed are transformed mitochondria. I must agree with phrussi (1925) that mitochondria take no visible part in the production of he proteid yolk in *Drosophila*. However, I maintain they do transform into atty yolk. This is somewhat singular in view of the fact that a large majority of the workers in the field of vitellogenesis, as listed by MacBride and Hewer 1931), have found that the origin and synthesis of the fatty yolk somehow nvolve Golgi material. Among the insects, this has been found to be true by Nath and Mehta (1929), Nath and Mohan (1929), Nath (1929), and Gresson, 1929, 1931). More recently, Fahmy (1949) found in the desert snail, *Eremina desertorum*, that 'the elements of the Golgi clumps become loaded with an unsaturated fat'. However, fatty transformation on the part of mitochondria s not an unknown phenomenon. For example, Smith (1931) observed it in he hepatic cell of the white rats, and I have reported it in the uropygial glands of birds (1936).

Regarding Golgi material, I have no doubt that in oocytes and nurses of ater stages of development, a large number of the discrete granules really contain a light centre, making each granule a Golgi vesicle. Even disregarding what has been observed of Golgi material in the oocytes of the other insects cited above, the behaviour of the Golgi bodies in the cells of certain larval glandular tissues in *Drosophila* (Hsu, 1947, 1948) inclines me to believe that,

in the present case, the light centre of each Golgi body must be a granule of secretory product of some kind, forming perhaps what may be regarded as a third type of yolk in *Drosophila* eggs.

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Some Effects of Abnormal Tonicity on Dividing Cells in Chick Tissue Cultures

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With two plates (figs. 2 and 3)

Summary

1. Chick tissue cultures were treated with hypo- and hypertonic saline for periods up to 1 hour. Dividing cells are more sensitive than resting cells to abnormal tonicity.

2. The effects of this treatment on dividing cells closely resembles that of various mitotic inhibitors. Cells are arrested either in pre-prophase or in metaphase, in which the chromatid pairs are scattered throughout the cell. With hypotony, anaphase may be disturbed, and cleavage may be prevented.

3. There is partial recovery when cultures are returned to normal saline after this treatment. Cells re-enter mitosis. After hypotonic saline, inhibited metaphases may either regenerate a spindle, or their scattered chromosomes may reconstruct as irregular nuclei or as separate chromosomal vesicles.

4. These results may have some bearing both on the course of normal mitosis and on the action of mitotic inhibitors.

Contents

Introduction

IN this paper are described the effects of adding to chick tissue cultures salines of abnormal tonicity for short periods. On dividing cells, the effect of such treatment is found closely to resemble the action of a number of mitotic inhibitors applied to the same material under the same conditions

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which have been described in previous papers (Hughes, 1949, 1950, 1952b). This correspondence of effect is sufficiently close to suggest that there is something in common between the action on the dividing cell of chemical inhibitors and of hypo- and hypertonic saline.

One difference, however, between the two types of treatment is that the normal environment of the tissue is more readily restored after disturbance of the tonicity of the medium than after the application of chemical substances. The results of returning cultures to a medium of normal osmotic pressure have been studied, and the regenerative changes within their cells may shed light on some of the normal events of mitosis in dividing cells.

Methods

Cultures of chick frontal bone were used similar to those previously employed for inhibitory studies, both in their source and in methods of cultivation (Hughes, 1949). The same methods of observation of living cells were again used. The experimental saline could be added to the culture during the progress of observation and of film recording of the changes within one cell under the phase microscope.

At the end of the period of observation, cultures were fixed either in Zenker's or Maximow's fluid, and were subsequently stained in Ehrlich's haematoxylin. Where the effect of treatment on batches of cultures was being studied, saline was added to them, usually for periods of 30 minutes, followed by fixation and staining. Nearly 300 cultures were used in the course of this work.

'Hypertonic Tyrode' was prepared by adding extra sodium chloride to the standard saline. In experiments on hypotonicity, Tyrode was diluted either with water, or with a solution containing all its constituents except the sodium chloride; this will be referred to as 'hypotonic Tyrode' ('H.T.'). In the following pages, percentage figures such as '10 per cent. Tyrode' relate to the content of NaCl compared with that of the standard saline.

The freezing-point of Tyrode is $-0{\cdot}62^{\circ}$ C. (Parker, 1938). It contains 8 gm. of NaCl per litre, which, calculation shows, is responsible for $0{\cdot}46^{\circ}$ C. of this depression. The freezing-points of the various dilutions of Tyrode with H.T. and with water given in fig. 1 are calculated on this basis. For this purpose it is sufficiently accurate to assume a linear relationship between NaCl concentration and freezing-point depression over the range of these dilutions. These figures do not allow for the effect of the salts present in the medium of the tissue culture, the volume of which is an appreciable though unknown fraction of that of the added saline. It is probably less than 10 per cent. of the whole.

Observations

With hypotonic saline

(A) *Effects on intermitotic cells.* As seen in the fixed cultures, the accumulation of vacuoles within the cytoplasm is the main effect of hypotonic treatment

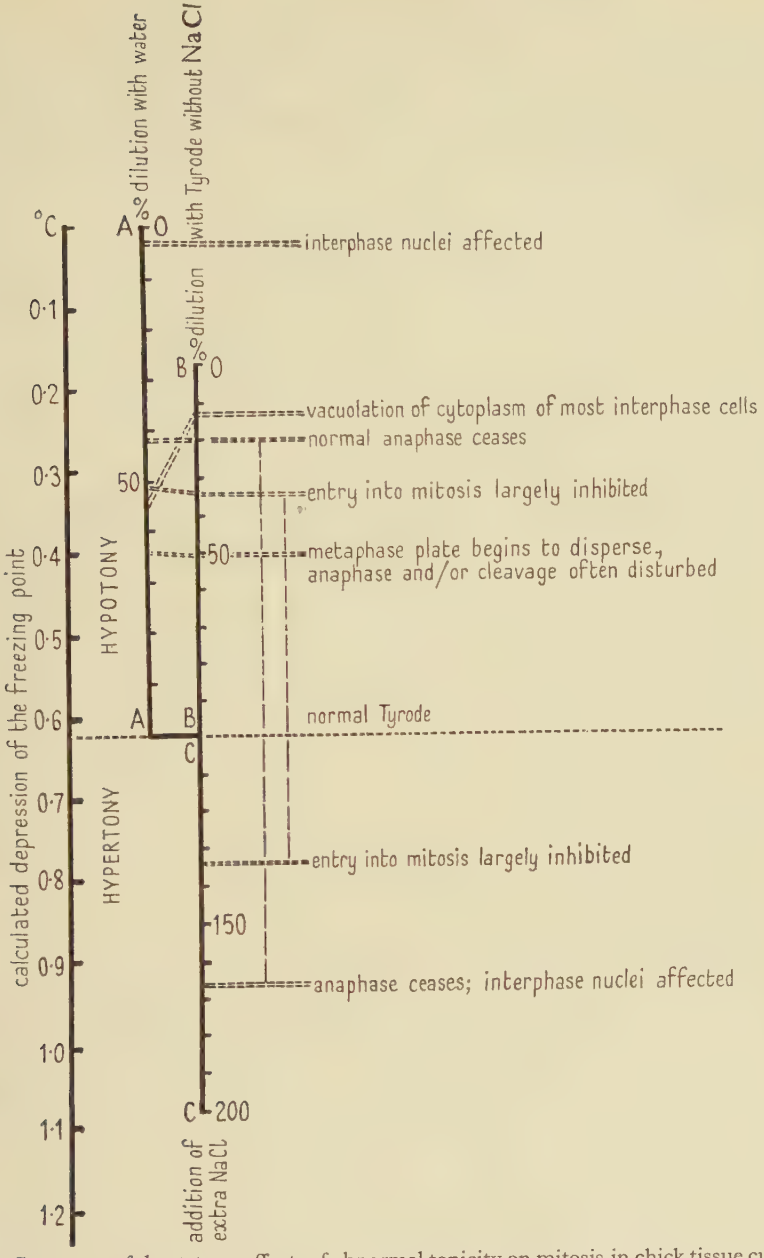

FIG. 1. Summary of short-term effects of abnormal tonicity on mitosis in chick tissue culture. The scales AA, BB, and CC are to be read according to the following examples:

AA. The mark denoting 40 per cent. indicates that 40 volumes of normal Tyrode solution were added to 60 volumes of distilled water.

BB. The mark denoting 40 per cent. indicates that 40 volumes of normal Tyrode solution were added to 60 volumes of a special Tyrode solution made up without sodium chloride ('hypotonic Tyrode').

CC. The mark denoting 140 per cent. indicates that sufficient sodium chloride crystals were added to normal Tyrode solution to bring the concentration of sodium chloride to 40 per cent. above the concentration in normal Tyrode solution.

on the cytoplasm of intermitotic cells. In untreated cultures, cytoplasmic vacuoles are uncommon during the first day after sub-cultivation, though they become more frequent later. They appear after treatment of cultures for 30 minutes with Tyrode diluted to 20 per cent. with H.T., though they are then still occasional and moderate in size. At 5 per cent. they are much larger and more frequent. Little further effect is seen when a culture is treated with H.T. alone.

When the Tyrode is diluted with water, however, small vacuoles appear at 60 per cent. and become as large and as common at 30 per cent. as with H.T. alone. With 3 per cent. Tyrode in water, the nuclei of intermitotic cells are affected. The nucleoli break up or even disappear and the nuclear membrane becomes indented by the adjacent large vacuoles in the cytoplasm. No signs of swelling of the whole nucleus were seen either at this dilution or in water alone. Such effects on intermitotic nuclei in cultures treated with water have already been described by Zollinger (1948).

(B) *Inhibition of cells from entry into prophase.* Early prophases become rare in the outgrowths of cultures treated with Tyrode diluted to 50 per cent. with water, or below 35 per cent. with H.T. Four or less may then be found in an outgrowth which normally contains one or more score. Occasional early prophases are still found at even lower tonicities. The number of prophases in cells treated with such hypotonic media falls gradually during the 30-minute period of treatment; occasionally there may still be found a dozen prophases with nucleoli after 20 minutes' treatment with 10 per cent. Tyrode in H.T. The duration of prophase in normal chick cultures is about 20 minutes (Hughes and Fell, 1949; Hughes, 1949). It is thus probable that once a cell has entered mitosis in the presence of a saline of low tonicity, prophase proceeds at the normal rate, but that within a few minutes of its addition, few further cells are then able to enter mitosis.

(C) *Arrest of cells in mitosis by osmotic swelling.* Dividing cells swell osmotically much more readily than do intermitotic cells. Hypotonicity can cause mitotic figures at all stages to swell, though more readily after the dissolution of the nuclear membrane than before. To produce swollen anaphases it is necessary to treat a culture with a saline of low tonicity for a period as short as 10 minutes (fig. 3D), for after that time the spindle of cells in metaphase is sufficiently swollen and disorientated to prevent anaphase movement. Normal early anaphases cease in cultures treated for 30 minutes with Tyrode diluted to 20 per cent. with H.T. or to 40 per cent. with water alone. After 15 minutes of treatment with salines of low tonicity, the majority of cells in mitosis in a culture are arrested at various stages of metaphase. They are greatly enlarged, and their paired chromatids are scattered throughout the cell (fig. 3B). The stage of development of the chromosomes corresponds to that at which mitosis was arrested; they are in various degrees of contraction in different cells. When greatly contracted, the long chromosomes are greatly constricted at the centromere (fig. 3C).

Such cells in which the pairs of metaphase chromatids are dispersed pro-

ide very favourable objects for the study and counting of the chromosomes. Hypotonic swelling might thus provide a further general method for this urpose. The chromosomes of the chick are well known as one of the most ifficult series to study, since many of them are so very small (White, 1932). Vhite counted from 61 to 66 chromosomes in embryonic metaphases; a milar range was here found in cells in culture swollen by hypotonicity. ccording to this author there are two large pairs of chromosomes with sub-ıedian centromeres; one pair of these are the sex chromosomes, of which nly one is present in the female. Study of the various batches of cultures repared in the course of this work suggests that those derived from embryos f either sex can be distinguished in this way.

The degree to which the chromatid pairs are dispersed by hypotonic treat-ıent increases with the extent to which the Tyrode is diluted. The effect is rst detectable at about 50 per cent. when H.T. is used as the diluent. Down to bout 30 per cent. dilution, the chromatid pairs are still found in the middle f the cell, although farther apart from each other than in the normal meta-hase plate. Normal early anaphases are still seen in such cultures, and it hus appears that some degree of swelling of the spindle is still compatible vith anaphase movement. At 20 per cent. dilution and below, however, the vhole metaphase plate is completely dispersed, and the chromatid pairs may e dispersed throughout the cell, particularly when H.T. is used as the diluent, or with water, vacuolation of the cell restricts the scattering of the chromo-omes. Dilution beyond 10 per cent. with H.T. does not cause further lispersal.

TABLE I. *Effect of hypotonic saline* (50–66 *per cent. Tyrode*) *on dividing cells in cultures observed in life*

Effect	*No. of examples*
Metaphase, normal anaphase and cleavage	3
Metaphase, normal anaphase, but no cleavage	2
No metaphase; pseudo-anaphase and cleavage	3
No metaphase, anaphase or cleavage	2
Arrest in metaphase	1
TOTAL	11

(D) *Nuclear reconstruction without a normal anaphase or cleavage of the cell.* Mitotic abnormalities of this kind were first seen in cultures under hypotonic reatment where cells at stages from late prophase to metaphase were followed vith the time-lapse camera (fig. 2). In these experiments, Tyrode was liluted to 50 per cent. or 66 per cent. with water. Eleven such film records vere made, and were subsequently studied in detail. The results of their nalysis are given in Table I. Nuclear reconstruction occurred in all cells xcept one, which was fixed too early to allow this to take place.

The formation of a binucleate cell by the suppression of cleavage is a well known mitotic abnormality (fig. 2, G–K). It is readily provoked in chick cultures by amino-purines or by the analogous compound benzimidazol (Hughes, 1952b). The second abnormality which has here been observed however, as far as I am aware has not previously been described. Immediately after the end of prophase, when the chromosomes already consist of paired chromatids, these collect in two groups at opposite ends of the nuclear area and then slowly move farther away from each other. These groups of chromatids then reconstruct into irregularly shaped nuclei, either with or without division of the cell (fig. 2, A–F). Close study of the film records was needed before this interpretation of these abnormal 'anaphases' could be accepted. The absence of metaphase, so conspicuous a stage in normal mitosis, suggested a distinct and unusual aberration; proof of this interpretation was seen in fixed cultures which had been similarly treated where cells were seen in which two unequal groups of obviously paired chromatids at opposite ends were separated by a cleavage furrow (fig. 3F).

Among the nine cells of this group which were followed through mitoses of varying abnormality as far as nuclear reconstruction, the absence of metaphase in five is correlated in each with an irregular nuclear outline after reconstruction. In cultures fixed after hypotonic treatment one can thus surmise that daughter nuclei of irregular outline are the result of such 'aploid' divisions.

In a specially clear example, the anaphase-like movement of such unequal groups of paired chromatids was plotted, and is here shown in fig. 4 along with an average normal anaphase curve reproduced from Hughes and Swann (1948). The extent of the movement and also its velocity is less than in normal

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Fig. 2 (Plate). Figs. 2 and 3 are photomicrographs of cells in cultures of chick bone which were filmed by phase contrast during treatment with hypotonic saline (Tyrode diluted to 66 per cent. with water) and then photographed again after fixation and staining with Ehrlich's haematoxylin. The times given are those between the application of the agent and photography. × 1840.

A–F. Effect of hypotonic saline on a cell in prophase.

A. 3 minutes after addition of saline. Late prophase.
B. 5 minutes. Two groups of paired chromatids separating in a pseudo-anaphase. (See fig. 4.)
C. 11½ minutes. Cleavage begins.
D. 16 minutes.
E. 23 minutes. Daughter cells are flattening.
F. Fixation after 28 minutes. The group of chromatids in each cell has reconstructed into a nucleus of irregular shape.

G–K. Effect of hypotonic Tyrode on a cell in metaphase.

G. 5 minutes after addition of saline.
H. 17 minutes. Cell in true anaphase.
I. 22 minutes. Late anaphase. Bubbling at surface, but no cleavage.
J. 46 minutes. Cell has flattened. Two normal daughter nuclei have reconstructed within.
K. Fixation after 55 minutes.

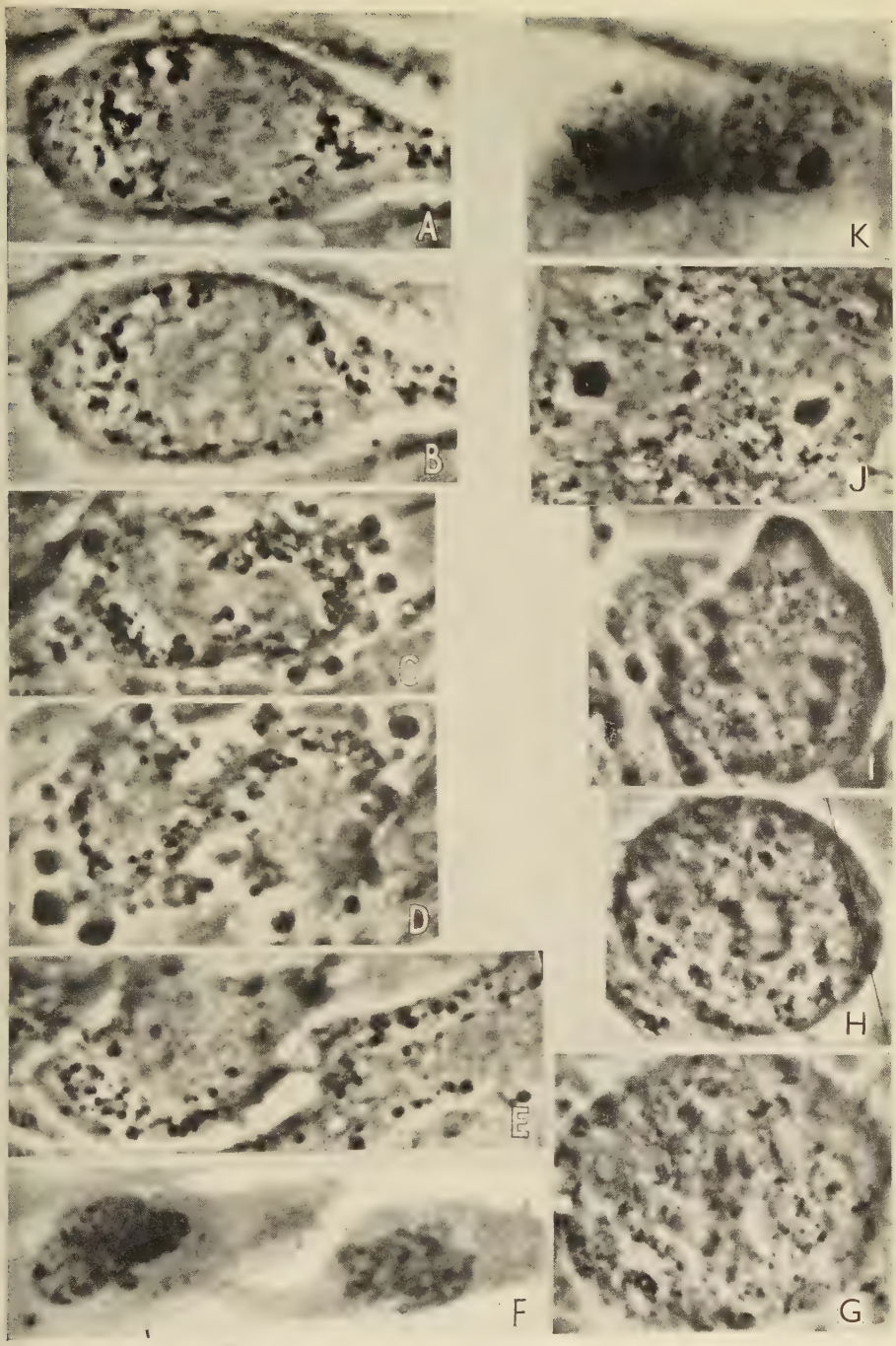

FIG. 2

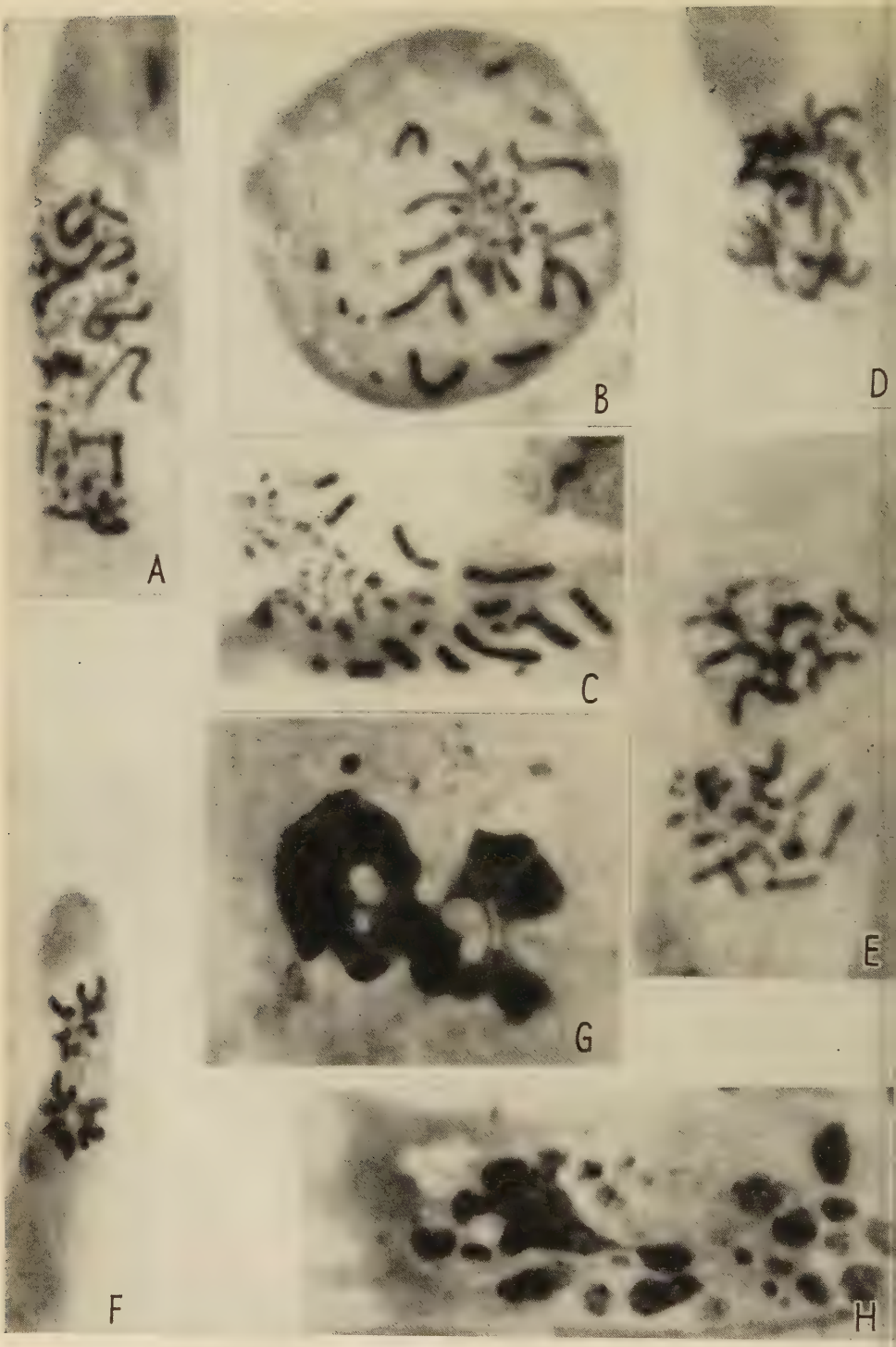

FIG. 3

A. HUGHES

anaphase; in this example cleavage begins later than normally, and its duration is greatly prolonged.

These mitotic abnormalities are observed in cultures under comparatively

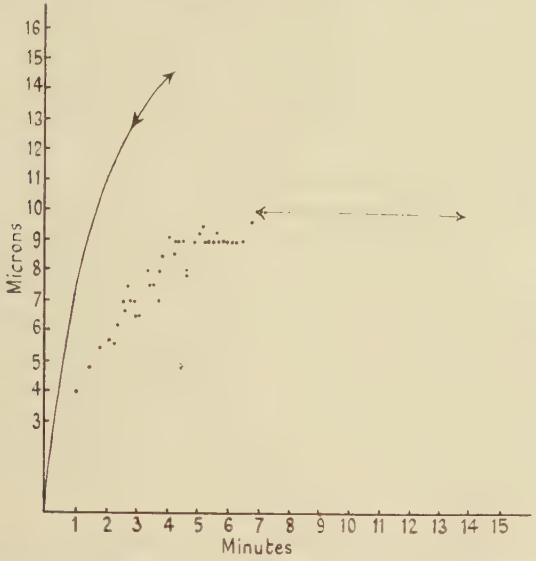

FIG. 4. Average normal anaphase curve of chick osteoblasts compared with an example of pseudo-anaphase movement of chromatid pairs under hypotonic treatment. The signs (< >) mark the beginning and end of cleavage.

mild hypotonic treatment where little dispersion of the metaphase plate is found. The spindle is presumably sufficiently disorientated to disturb the normal equal centromeric attachment of the chromatids, but not enough to inhibit the poleward movement of the abnormally attached chromatid pairs. As this occurs prematurely with the omission of metaphase, the centromeric attachments may normally be equalized during this period of mitosis.

FIG. 3 (Plate). Photomicrographs of cell in mitosis in chick cultures treated with hypotonic saline. A–F, fixed after treatment; G–H, returned to normal Tyrode before fixation. Ehrlich's haematoxylin. ×2500 (except F).

A. Effect of 10 per cent. Tyrode for 10 minutes on a cell in prophase.
B. Scattering of paired chromatids in a cell in metaphase treated for 30 minutes with 10 per cent. Tyrode.
C. Similar treatment and effect to B, except that the chromatids are strongly contracted.
D. Effect of 10 per cent. Tyrode for 10 minutes on a cell in early anaphase.
E. Effect of 10 per cent. Tyrode for 10 minutes on a cell in telophase. Cleavage of the cell has been inhibited.
F. From a culture treated with 60 per cent. Tyrode for 44 minutes. A cleavage furrow is separating two unequal groups of paired chromatids. ×1800.
G and H. Cells from a culture treated for 15 minutes with 10 per cent. Tyrode, and then with normal Tyrode for 30 minutes. Scattered metaphase chromosomes have reconstructed either into an irregular nucleus (G), or into separate chromosomal vesicles (H).

(E) *The effect of normal Tyrode on cultures previously submitted to hypotonic treatment.* When cultures have been treated with Tyrode sufficiently diluted to arrest dividing cells in metaphase and to disperse the chromatids, and the incubation of these cultures is continued for 1 hour after the withdrawal of the saline, the great majority of the arrested metaphases remain in this condition except that the swollen cell becomes more heavily vacuolated. If, however, after the diluted saline has been withdrawn, it is replaced by normal Tyrode, a number of different changes may occur in the cells of the outgrowth. In the experiments to which this section refers the preliminary hypotonic treatment was with 10 per cent. Tyrode for 15 minutes. The cultures were then returned to normal Tyrode for periods of 15 minutes to 1 hour. In such cultures the effects of the return to normal tonicity may be described as follows.

A few cells undergo degeneration; some of these seem to fragment explosively, others shrink while the nucleus shrivels and becomes pycnotic. Most cells, however, remain viable. The vacuolation of the cytoplasm is much less conspicuous 15 minutes after the return to normal tonicity, and by then all the arrested metaphases have shrunk very markedly. After about 30 minutes, cells once more begin to enter prophase, though the number of cells in this stage of mitosis remains generally below those in untreated cultures.

The arrested and dispersed metaphases may react in any of three ways:

(*a*) The spindle may be regenerated, and a normal mitosis resumed.

(*b*) The dispersed chromatid pairs may reconstruct, either individually into separate chromosomal vesicles (fig. 3H) or into one or more nuclei of irregular or lobulated outline (fig. 3G).

(*c*) In arrested metaphases in which neither (*a*) nor (*b*) occurs, the chromosomes lose their normal form, while retaining their intensity of staining. Usually, but not always, they aggregate together into dense masses. This reaction seems to result from some irreversible change in their nucleoproteins, for no form of nuclear reconstruction then seems to follow.

The proportion of cells in arrested metaphase in each culture which undergo these three possible fates seems to bear no relation to variations in the details of the experiment, such as the time of treatment with hypotonic and normal saline, and the degree of dilution of the Tyrode in the first phase. In the individual cultures of a batch given uniform treatment there may well be considerable diversity in result.

Fifteen minutes after the return of a culture to normal tonicity, cells with scattered pairs of unchanged chromatids are no longer to be seen. Where the chromosomes have remained dispersed they are then all in reconstruction, and are forming either separate chromosomal vesicles, or lobulated nuclei. In other viable cells at the same stage, the chromatid pairs have drawn together, and such cells are almost indistinguishable from normal metaphases.

After a further 15 minutes the most conspicuous stages of mitosis are ana-

phases and telophases. These are present in much greater numbers than were prophases 15 minutes earlier; it is thus probable that most of these cells now in late stages of mitosis had previously been arrested in metaphase, and that in them the mitotic spindle has been regenerated. The reappearance of the mitotic spindle under these conditions has already been described by M. R. Lewis (1934).

Between 30 minutes and 1 hour after the return of cells to normal tonicity, the cells still in arrested metaphase in which reaction (*c*) has occurred become more conspicuous. In them, the chromosomes are usually widely scattered, but consist merely of rounded, densely staining granules. It seems that these cells represent a fresh crop of arrested 'metaphases', and that by then irreversible changes have occurred in the cultures which render some cells which enter division incapable of completing a normal mitosis.

In one experiment, cultures were treated first with 10 per cent. Tyrode for 15 minutes, which was then replaced with less diluted Tyrode. They were then fixed after a further 30 minutes. After this further period in 20 per cent. Tyrode, the great majority of arrested metaphases were unchanged, though occasional reconstructions were to be seen. When 40 per cent. Tyrode was used, both normal anaphases and telophases were present, and also instances of reconstruction without cleavage, or without anaphase as well. Reactions (*a*) and (*b*) seemed to enter at the same point in this series. After 60 per cent. Tyrode normal metaphases were much more common than those which still remained in the inhibited state. Prophases did not reappear in any numbers until 80 per cent. Tyrode was used.

With hypertonic saline

(A) *General remarks.* Tyrode saline was prepared containing amounts of NaCl greater than the normal, the most concentrated having twice the usual amount. Batches of cultures were treated with these salines for periods of 30 minutes, and were then fixed and subsequently stained. At 125 per cent. no effect was noticeable; at 133 per cent. prophases became very rare in the treated cultures. They disappeared completely at 150 per cent. At this concentration, normal anaphases and telophases are reduced in numbers to one or two in each culture; frequently cells in metaphase show adverse changes. The chromosomes are mostly clumped together and envelop the spindle, which is shrunken transversly. The whole then forms a narrow and somewhat irregular densely staining body lying in the long axis of the cell. At 166 per cent. in this series, normal anaphases have disappeared, and the intermitotic cells show some effect of the hypertonic treatment in that the chromonemata are abnormally distinct. Such nuclei could not be distinguished from very early prophases, had any cells in this stage been present. At 200 per cent. all intermitotic nuclei are grossly abnormal. Their chromonemata are in the form of short rods or granules, which stain more deeply with Ehrlich's haematoxylin than do the nucleoli.

The effect of hypertonic treatment of such cultures is variable, for in a

further similar experiment 150 per cent. Tyrode was sufficient to affect most of the intermitotic nuclei.

(B) *The effect of return to normal Tyrode after hypertonic saline.* Batches of cultures were treated first with 150 per cent. Tyrode. Some were fixed at this stage, and the rest were incubated in normal Tyrode for periods of 30 minutes or 1 hour. Those cultures fixed after hypotonic treatment alone showed that in this experiment its effects corresponded with those described in the foregoing section. In these particular cultures the only normal mitotic figures were a few late telophases.

Half an hour after the subsequent return to normal tonicity, prophases reappear. After an hour, all stages of mitosis are seen, and in some cultures, anaphases and telophases are especially prominent. A large proportion of these later stages of mitosis must have come from the cells seen in prophase at the earlier period. Possibly others are derived from metaphases inhibited by hypertonicity in which the spindle may later have resumed its normal function. It is clear, however, that the spindle is more readily regenerated after hypotonic treatment, for then anaphases and telophases reappear before prophases, whereas the reverse sequence is seen after hypertony. Some cells inhibited in metaphase by this treatment are clearly unable to recover even after 1 hour in normal saline. Cells in which chromosomes of blurred outline are scattered throughout the cytoplasm are then still common.

Nuclear reconstruction without anaphase or cleavage after hypertonic treatment seems to occur very rarely, if at all.

Discussion

There are two main points of similarity in the action on tissue cultures of salines of abnormal tonicity and of mitotic inhibitors:

1. Dividing cells are more sensitive than those in interphase.
2. Arrest of cells in division tends to occur at the same points in the mitotic cycle.

1. The sensitivity of the dividing cell to various influences has been demonstrated many times, but less often have both osmotic and chemical effects been investigated at the same time on one type of cell, as for instance in Herlant's well-known study on the egg of *Paracentrotus* (Herlant, 1920).

It is possible that the greater susceptibility of the dividing cell to osmotic swelling and to the effects of chemical inhibitors is related to a weakening of the cell membrane during mitosis, resulting in a greater permeability. That this cannot be the only general change within the cell is sufficiently demonstrated by the increased sensitivity of the dividing cell to radiation. All of these must be related to some more fundamental events whose nature is still largely obscure. It may well be, however, that the macromolecular changes at the cell membrane are less difficult to study than those within the cytoplasm, and may shed some light upon both. One difference between the reaction to hypotonicity of chick cells in mitosis and in interphase which the

present work suggests is that the effect of the balanced concentration of cations in a saline such as Tyrode in controlling cell permeability is much less marked than in the dividing cell. Fig. 1 shows that the various inhibitory effects of hypotonicity on dividing cells are a function of the tonicity of the solution, and at each value are not increased by reduction of the concentration of cations other than sodium below the normal values when Tyrode is diluted with water alone. On the other hand, the extent to which the cytoplasm of intermitotic cells is vacuolated is very much greater when Tyrode is diluted with water than with H.T. Further comparison of the effects of ionic balance on cells during and between division could be made more effectively on marine eggs where the composition of the environment is more closely under control than in a tissue culture. It does not appear that any of them have yet been studied from this point of view.

2. The mitotic cycle of the chick cells in culture may be inhibited at two main points, namely, before prophase, and before anaphase. Cleavage and nuclear reconstruction may also be affected by some chemical agents (Hughes 1949, 1950). Once a cell has entered prophase, while the nuclear membrane is still present, it is at the least susceptible point of the cycle. Both in hypotonicity and in hypertonicity, prophases cease earlier than do normal anaphases with increasing deviation from normal tonicity. Chemical inhibitors are more specific, and those of the colchicine type which primarily affect the spindle have little or no effect on entry into prophase.

The sensitivity before prophase to fluoride and iodoacetate and also to hypotonicity and cold suggests a parallel with the effect of the same agents on the escape of potassium from mammalian erythrocytes in the 'pre-haemolytic state' (Hughes, 1950; 1952a, p. 185). This parallel can hardly be extended to the effects of hypertonicity on the two types of cell, for in the cat erythrocyte, this condition causes a decrease in permeability to potassium and an increase in that to sodium ions (Davson, 1940). These two monovalent ions also move in opposite ways at low temperatures, for in human blood sodium is then taken up as potassium is lost (Flynn and Maizels, 1949).

It is possible that the differences in the effect of hypo- and hypertonicity on dividing cells in cultures and in the course of their subsequent recovery on return to normal tonicity may be related to different types of disturbance of the normal distribution of cations within the cell. Other changes within the cell must also be involved, however, and of these a shift in the normal equilibria between the cell proteins and water is clearly one. Within the mitotic spindle the gross effect of changes in water balance is clearly visible. In hypertonicity the metaphase spindle shrinks equatorially, as Bělař showed in the grasshopper spermatocyte (Bělař, 1929). The swelling of the spindle in hypotonicity is shown when first the chromatid pairs on the metaphase plate move slightly apart. When this process is carried further, the spindle presumably is largely or wholly disorientated, though its regeneration on return to normal tonicity shows that the orientating centres at the spindle poles and on the chromosomes are still functional. It may well be that the inhibition of

cleavage in hypotonicity is due to the swelling and dispersal of the cortical gel which probably plays the same part in the cleavage of a fibroblastic cell as in a sea-urchin egg (Hughes, 1952, p. 144).

Whatever the changes within the cell which are induced by abnormal tonicity, their similarity in gross effect with those of various inhibitors suggests that there must be some common factors in the action of both types of agent on cells in mitosis. A cell inhibited in metaphase by hypotonicity closely resembles one affected by colchicine where the chromosomes have not clumped together (Ludford, 1936). Where the chromosomes themselves are apparently irreversibly affected by abnormal tonicity, and are degraded to densely staining granules of chromatin, such effects closely resemble those of the 'radiomimetic poisons' such as mustard gas, the action of which on chick cells has been described by Hughes and Fell (1949).

Some of the observations in the present work on the effects of the return of cultures to their normal tonicity after hypotonic treatment may shed a little light on the normal events of telophase. When a cell in metaphase in which the spindle has been disorientated by hypotonicity is returned to normal Tyrode, the scattered chromatid pairs may at once begin nuclear reconstruction. If, however, the spindle is regenerated, this process is delayed until anaphase movement of the chromosomes is completed. By the end of anaphase the spindle is disorientated; so in neither instance does nuclear reconstruction occur in the presence of a spindle. The reappearance of a spindle in the treated culture thus delays reconstruction. However, in a cell inhibited in metaphase by hypotonicity, although no spindle is present, this event does not occur, and the chromatid pairs remain unchanged until some stimulus is given which results from the return to normal tonicity. At the end of a normal anaphase there must occur some comparable change, separate from the disorientation of the spindle, but held in abeyance until this structure is dispersed.

One effect of returning a cell from an environment of low osmotic pressure to one of the normal value is the withdrawal of excess water. This would suggest that water may normally be lost from a cell in telophase; the bubbling at the surface of a fibroblastic cell at this period is sometimes so regarded, though without any direct evidence. It is certainly possible to dissociate bubbling from nuclear reconstruction, as for instance by treatment with sulphydryl reactants (Hughes, 1949, 1950). The fact remains, however, that withdrawal of water from a swollen cell may lead to reconstruction without anaphase, whereas there is no indication that the reverse change after hypertonic treatment can evoke the same reaction.

The swelling of the daughter chromosome groups in telophase suggests an imbibition of water (Fell and Hughes, 1949). If this is so, the process should be arrested by hypertonicity. Such an effect has been demonstrated in the staminal hair of *Tradescantia* (Shigenaga, 1929), but not, as far as I am aware, in any animal cell. There is no suggestion of an inhibition of nuclear reconstruction by hypertonicity either in the sea-urchin egg (Gray, 1913) or in Amphibian cells (Aisenberg, 1935) or in the present work on chick cultures.

This evidence, then, so far as it goes, is consistent with Caspersson's view that the swelling of the early telophase nucleus in grasshopper spermatocytes is due to the synthesis of protein therein (Caspersson, 1950, p. 86). Moreover, it is at this point in the mitotic cycle that Lison and Pasteels assert that deoxyribonucleoproteins are being synthesized within the nucleus (Lison and Pasteels, 1951). If telophase is a period of active synthesis within the nucleus, the sensitivity of this phase of mitosis to sulphydryl reactants in chick cells may well be related thereto. It would be a notable gain in mitotic physiology if the significance of this aspect of telophase were clearly demonstrated.

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Modes of Gastrulation in Vertebrates

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Summary

The modes of gastrulation in the different classes of Vertebrates are discussed from a comparative point of view, with the particular aim of finding some system which avoids postulating a sharp dualism between the anamniote telolecithal forms, in which the edge of the blastoderm consists of presumptive endoderm, and those of the Amniotes, in which it is ectodermal. It is suggested that the most profitable way of regarding all blastodermic forms is to envisage them as derived from the basic type of blastula by a process in which the roof and floor are squeezed together, so that a flattish double-layered plate is produced. The concept of the 'primitive edge' of such a blastoderm is introduced, and it is suggested that in the amniotes the presumptive ectoderm has expanded greatly beyond the primitive edge, while there has also been a strong convergence towards the dorsal side. The conditions in certain highly yolky amphibian eggs are shortly described, and it is argued that they show conditions similar to those which would be expected on the hypothesis of a squeezing together of the blastula roof and floor.

IT is during gastrulation that the fundamental plan of the vertebrate body is brought into existence, and one of the most important tasks of comparative anatomy is therefore to understand the relations between the various modes which this process follows in the different groups. As Pasteels (1940) has pointed out, the use of vital-stained marks and of time-lapse cinematography forces us nowadays to envisage gastrulation as a dynamic process, and renders out of date the attempt to set up a series of static forms (such as the classical blastula and gastrula, or the 'Gastraea' of Haeckel) through modifications of which all vertebrate embryos must pass. Instead of this, we should now discuss the relations between the dynamic systems themselves. This is most easily done by considering each gastrulating system in terms of, firstly, the map of presumptive areas just before gastrulation begins, and, secondly, the set of movements by which the areas on such a map reach their final positions. The newer and more thorough methods of investigation have as yet only been applied to a comparatively small number of types, but in spite of the large gaps in our knowledge, we already have a sufficient basis to erect a tentative scheme for the major vertebrate groups. It is particularly to the Belgian embryologists Dalcq (1938) and Pasteels (1940) that we owe the first attempts to formulate this.

By far the best-known map of presumptive areas is, of course, that of the moderately yolky Amphibia, which were the first material to which the vital staining technique was applied by Vogt (1929). In the early stages of such

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studies there was inevitably a tendency to take this as a central type, and the maps for other Vertebrate groups as variations of it. Strictly speaking, this is unjustifiable, since the Amphibia are not a particularly primitive type of vertebrate. In practice, however, such a procedure is probably not as dangerous as might appear. For one thing, the amphibian egg is only moderately yolky, and the primitive arrangement of the areas has not been greatly distorted by the presence of an enormous store of deutoplasm, as it has both in most of the more primitive fishes and in the more advanced reptiles and birds. Moreover our rather scanty knowledge of the disposition of presumptive areas in the most primitive vertebrates agrees fairly well with the amphibian plan. In particular, the map in the prochordates, as worked out by Conklin (1905), Berrill (1945), and Vanderbroek (1938) for the ascidians and *Amphioxus*, very closely resembles it. We therefore seem justified in postulating a primitive vertebrate blastula, containing rather little yolk, as a sphere consisting of an upper cap of ectoderm separated by a ring of mesoderm from a lower cap of endoderm, within which the position of the blastopore lies somewhat to one side in the dorsal plane; the areas of the axial organs, chorda, and neural plate are concentrated towards this plane in front of the blastopore.

The classical amphibian plan, characteristic of such forms as *Triturus* or axolotl, can be derived from this basic map merely by increasing the amount of yolk contained in the vegetative cells. The main problem of comparative vertebrate gastrulation is to formulate a scheme which will accommodate both the blastodermic types of development, seen in the teleosts and Selachia on the one hand and the birds and reptiles on the other. The maps of presumptive areas in these forms differ sharply in the fundamental fact that in the former the edge of the blastoderm is constituted by presumptive endoderm, while in the latter it is presumptive ectoderm. What may be perhaps regarded as the present orthodox view, represented by Pasteels (1940), accepts this duality as irreconcilable, and supposes that the two meroblastic types have been derived in two quite different ways. It suggests—to put matters in the simplest possible terms—that the fish type of blastoderm could be derived by opening the primitive map at some point within the endoderm (near the site of the blastopore) and inserting there a mass of undivided yolk; while the amniote scheme could be similarly derived by making an opening in the presumptive ectoderm, and inserting the yolk there (fig. 1).

Such a scheme would appear to be simple and convincing enough as it applies to the fish. We should indeed expect that if the yolk becomes segregated from the cellular material, it would take the form of a large unsegmented mass lying at the vegetative end of the egg, and would therefore be in immediate contact with a ring of endoderm. But the same considerations which render this convincing, make the suggestion for the amniotes seem artificial and unlikely. Moreover the scheme does not take account of the fact that in the higher amniotes, such as birds, the presumptive endoderm is from the beginning hidden in the depths of the blastoderm, underneath instead of surrounded by the other presumptive areas. It is the purpose of the present

discussion to argue that the Pasteels scheme deals too exclusively with the possible modifications of the basic map considered solely in terms of the surface of a sphere, and that a preferable alternative system can be derived if one pays more attention to the disposition of the areas in depth as well as on the surface (Waddington, 1952).

The scheme to be suggested may be characterized as one mainly of deformations rather than of the simple insertion of the yolk into a hole imagined

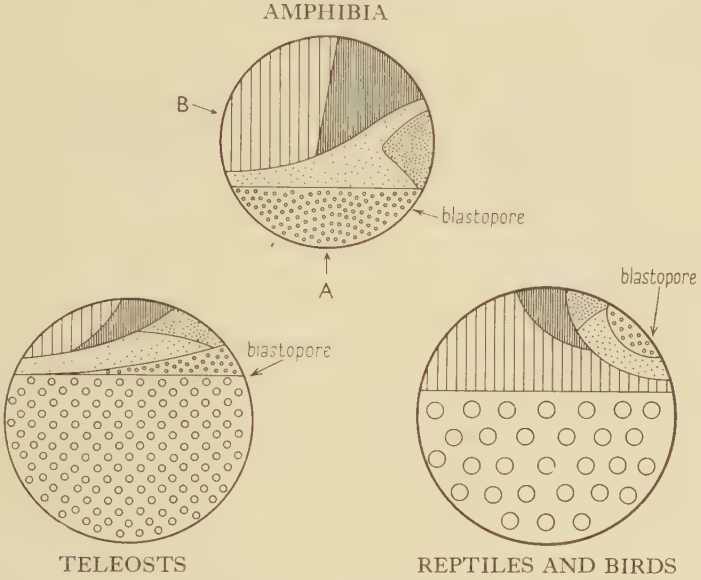

FIG. 1. Maps of the presumptive areas in blastulae, seen from the left side. In the upper figure, A indicates the place into which the yolk-mass would have to be inserted to produce the teleost condition, while B shows the place of insertion of the yolk-mass to produce the amniote arrangement.

Close lines, neural plate; spaced lines, epidermis; close dots, chorda; spaced dots, mesoderm; small circles, endoderm; large circles, yolk.

to be made in the basic plan. We shall consider the yolk always to occupy the most vegetable part of the egg, and we shall accept the Pasteels derivation of the maps of the Selachia and Teleosts, which does of course involve primarily an insertion of the yolk mass at this point; it is in relation to the Amniote plan that the consideration of deformation becomes of major importance.

In order to derive the maps for reptiles and birds from that for the Amphibia, it is suggested that we should envisage the mass of yolk as segregated from the cellular material, which is then imagined to be pressed down, from animal to vegetative, so that the original sphere forms a flat double-layered plate (fig. 2). The edge of this plate will run in some more or less latitudinal line around the basic spherical plan; it will be referred to as 'the primitive edge'. If it runs wholly above the presumptive endoderm, then none of that

area will appear on the upper surface of the plate; it will all be hidden in the lower layer. This appears to be the situation in birds (Pasteels 1937; Waddington 1932, 1952), and in some reptiles (Peter 1938), but in the Algerian turtle (*Clemmys leprosa*, Schw.) Pasteels (1937) has shown that some endoderm remains on the surface, and in this case the primitive edge must run rather lower.

Such a deformation will present us with a blastoderm which already has one major point of resemblance to the amniote plan, in that the majority or

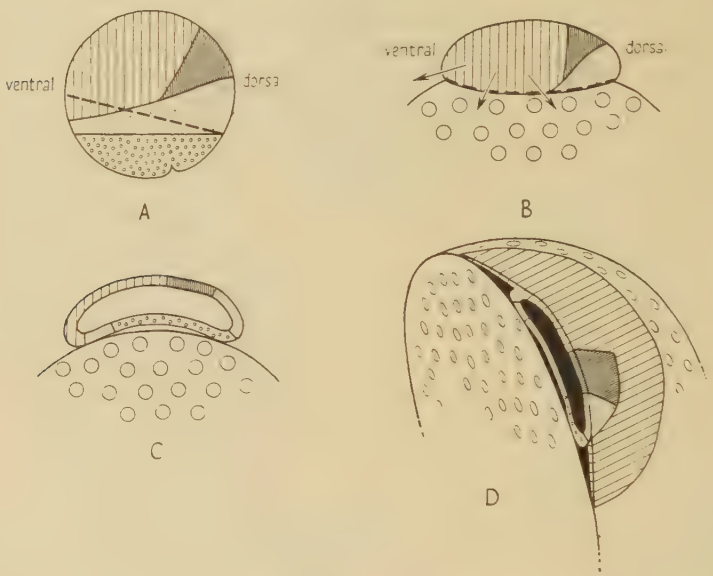

FIG. 2. A, the basic vertebrate blastula, with a line of folding (the 'primitive edge') indicated by a broken line. B, lateral view of the blastula, imagined as flattened out on the surface of a mass of yolk, having folded along the primitive edge. C, section of the same. D, dorso-lateral view of a halved blastoderm, which has been derived from that shown in B and C by the expansion of the epidermal area in the manner indicated by the arrows in B.

even the whole of the endoderm is already in a lower layer whence it can be derived by delamination rather than by any more elaborate process of invagination. The primitive edge of the blastoderm, however, in no way resembles the edge of the blastoderm as we know it in the bird's egg at the beginning of gastrulation; on the dorsal side it would be bounded by axial mesoderm, while more ventrally we could suppose either that it is bounded by lateral mesoderm or by ectoderm.

We may discuss the latter point first. There is no doubt that the ventral side of the amniote blastoderm is bordered by presumptive ectoderm, and there is every reason to believe that there is no presumptive mesoderm lying on the surface in this region. We have then two possible lines of argument.

ne would suppose that during the derivation of the amniote map the com-
lete ring of mesoderm which circles the basic vertebrate map has become
roken, the presumptive ectoderm coming into direct contact with the pre-
ımptive endoderm. Weissenberg (1934, 1936), who first applied the vital
ain method to the study of gastrulation in cyclostomes, supposed that this
as the case in that relatively primitive group, and if that were indeed so,
ıere would be nothing untoward in postulating a similar condition in the
ncestry of the amniotes. However, Pasteels (1940) has rather convincingly
rgued against Weissenberg's interpretation of the cyclostome situation, and
ıe hypothesis can therefore no longer find support in that group. But there
another similar case among the lower vertebrates. Vanderbroek's studies
1936) on the Selachian *Scyliorhinus* (*Scyllium*) *canicula* appear to demonstrate
onclusively that in that form (though probably not in all Selachia) there is
o presumptive mesoderm on the ventral side of the blastodisc, where the
ctoderm comes directly in contact with the presumptive endoderm.

The second line of derivation of the amniote map would, however, not rely
n any such supposition of a break in the original mesodermal ring, but
ould suggest that the ventral side of the primitive edge lies above the region
f ventral presumptive mesoderm, which therefore becomes located below
ıe surface.

We do not in fact know with any certainty whether in the bird embryo there
any presumptive mesoderm lying beneath the surface near the periphery
f the blastoderm at some distance from the site of the primitive streak.
lthough there is no doubt that some lateral mesoderm moves through the
reak, and originally occupies the region on the surface indicated for it in the
ıaps of Pasteels (1937) and Waddington (1932, 1952), there is no demonstra-
on that this is true of the whole non-axial mesoderm. Experimental results
ıch as those of Olivo (1928) and Rudnick (1938) show that mesoderm can
evelop from isolated peripheral fragments of the unincubated blastoderm,
nd although this development may be due to regulation (i.e. be *bedeutungs-
emde*), perhaps it is more likely than not that there is some presumptive
on-axial mesoderm beneath the surface in such regions.

There is a further point to be borne in mind. We have considered the
elation between the Amphibia and birds in terms of the maps of presumptive
reas. These record what does happen, but not what may happen; that is to
ay, they take no account of the state of determination of the germ. Now we
now that in the Amphibia the site of the blastopore is rather definitely
etermined soon after fertilization by the appearance of the grey crescent. In
irds, on the other hand, the position of mesoderm-invagination is not fixed
ntil a much later stage (Waddington, 1933; Lutz, 1949). The experimental
vidence shows quite clearly that the presumptively ventral region of the
vian blastula is quite capable of becoming the dorsal side, and thus of pro-
ucing axial mesoderm. The potentiality for forming mesoderm therefore
xtends all round the periphery of the blastoderm. Even if we reject the pos-
ibility, discussed in the last paragraph, that there is presumptive lateral

mesoderm located below the surface in regions far removed from the primitive streak, the fact that the mesodermal potentiality extends so widely could be considered to establish a certain parallelism with the uninterrupted mesodermal ring of the amphibian presumptive map.

The conditions at the dorsal side of the blastoderm require further consideration. There is no doubt that by the time the primitive streak is beginning to form in a bird (or the endoderm to invaginate in a reptile), the edge of the blastoderm is composed entirely of presumptive ectoderm, while the 'primitive edge', derived according to our scheme, would be formed on the dorsal side of presumptive mesoderm or endoderm. There are two deformations which it seems reasonable to postulate in order to reconcile these two situations. The first is the occurrence in the amniote blastoderm of a strong convergence towards the dorsal mid-line, the second an expansion of the presumptive ectoderm which carries it, as it were, well beyond the primitive edge (fig. 2, B and D). The convergence is still in progress when the primitive streak begins to appear (Wetzel 1929, Gräper 1929), at which time it is taking place just inside the edge, which is already ectodermal. We do not know for certain, however, whether this ectoderm has not itself been brought to the dorsal side by earlier converging movements of the same kind as later build up the streak. Whether this is so or not, we may legitimately call on such a movement as an element in the deformations by which, during the course of evolution, the amniote plan has been derived from the basic scheme.

An expansion of the ectoderm beyond the primitive edge might be expected on several grounds. It is, of course, characteristic of the amniote blastoderm itself during its expansion over the yolk. A similar movement is also involved in the epiboly which is so characteristic of gastrulation in the Amphibia. This is, of course, most marked during the process of gastrulation itself, when the presumptive ectoderm expands to cover the whole surface of the egg. In a form such as the tree frog *Rhacophorus* (fig. 3, C and D), a large part of the surface is originally covered by heavily yolk-laden endoderm cells, and the expansion of the ectoderm to cover these is very considerable. It takes place rather early during gastrulation, while the mesoderm mantle has not nearly finished forming a complete layer between the ectoderm and endoderm. In many, if not all, forms of Amphibia, the epiboly begins even earlier, before the appearance of the blastopore. What is being postulated here is that during the evolution of the amniotes, a similar but more extensive movement may be considered to have affected the disposition of the presumptive areas before gastrulation begins.

If one may postulate such an ectodermal expansion and dorsal convergence, and suppose that the dorsal part of the primitive edge sometimes lies higher (as in birds in which all the endoderm is in the lower layer) and sometimes lower (as in reptiles in which some endoderm may be on the surface), one can derive the amniote scheme without making the artificial assumption that the yolk is inserted into the presumptive ectoderm of the basic vertebrate plan. Moreover such a derivation makes more comprehensible the otherwise

surprising fact that in a form such as the bird the first mesoderm to be invaginated is not axial mesoderm, but material which will eventually form part of the lateral plates. It will be noted that in birds the primitive edge must lie, in the dorsal plane, well above the site of the blastopore of the basic scheme, which is within the endodermal area. It presumably runs more or less along the boundary between the presumptive endoderm and mesoderm. Some distance on each side of the dorsal midline, this becomes the position of the

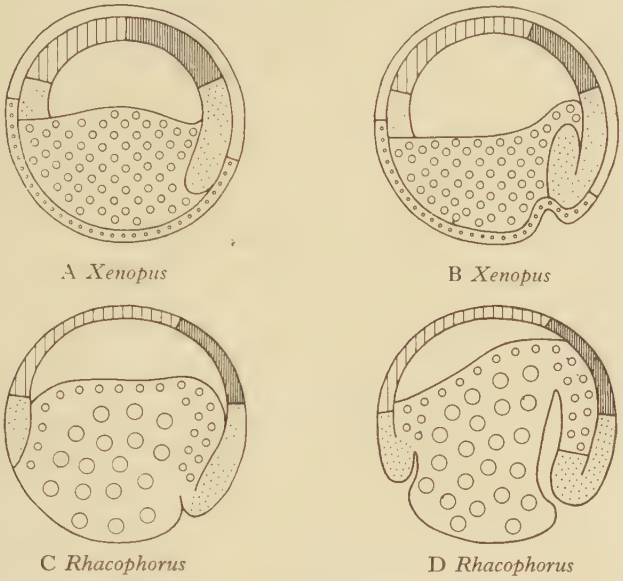

FIG. 3. A and B, longitudinal sections through blastula and mid-gastrula of *Xenopus*. Note the superficial layer, which forms the external linings of the ectoderm and endoderm; and the internal invagination in B. C and D, similar sections through early and mid-gastrulae of *Rhacophorus*.

lateral lips of the blastopore. It could therefore not be expected that it would necessarily be the most dorsal part of the original blastopore (from which axial mesoderm is formed) which would begin invaginating first in the bird; it is not so surprising to find that the invagination begins by involving material which is derived from more lateral parts of the basic plan, where the primitive edge runs nearest to the site of the blastopore lips.

It is instructive to consider the conditions in some of the highly yolky Amphibia, to see how far they present parallels to the various types of deformation which have been postulated as affecting the telolecithal amniotes. Pasteels (1949) and Nieuwkoop and Florschütz (1950) have recently described the process of gastrulation in the rather abnormally yolky eggs of the anuran *Xenopus laevis*. Some of the important points which they discovered are brought out in the diagrams on fig. 3, A and B. One may notice in the first

place that the outermost layer of tissue in the blastula consists solely of presumptive ectoderm and endoderm; it continues always to form the boundary between the embryo and a non-embryonic space, either the external medium or the gut-cavity. This rather peculiar state of affairs seems to suggest some parallel with the expansion of the presumptive ectoderm over the original primitive edge, which has been postulated in the amniote. Secondly, these authors draw attention to the extension of the presumptive mesoderm on the dorsal side down towards the site of the blastopore, so that it comes directly to overlie part of the endoderm. We have here conditions which strongly remind one of the folded dorsal side of the primitive edge in amniotes. The peculiar 'internal invagination' indicated in fig. 3B by which the inrolling of the mesoderm gets far ahead of the corresponding formation of the archenteron, seems to be a peculiarity of certain Anura with no particular connexion with anything in the amniotes.

The extension of the presumptive mesoderm downwards over the endoderm, so that the latter is from the beginning buried in the depths of the blastula, is even more clearly seen in two other yolky anuran eggs which I have recently studied, those of the Australian frog *Pseudophryne australiensis* and of a Ceylonese tree frog belonging to the genus *Rhacophorus*. In both these, but particularly in *Rhacophorus* (fig. 3 C and D), there is a very strong distinction between the small, cytoplasm-rich cells of the animal pole, and the large yolk-laden cells of the vegetative region, so that there might almost be said to be a blastoderm. The edge of this 'blastoderm' is thickened, and extends down over the yolky cells for a considerable distance, particularly on the dorsal side. When gastrulation begins the blastoderm extends very rapidly round the whole egg, so that in *Rhacophorus*, for instance, a yolk plug is formed which initially includes almost the whole of the vegetative hemisphere. The archenteron cuts right into the thickness of the mass of yolky cells which lie beneath the original thickened edge of the pseudo-blastoderm, and there seems no doubt that a good deal of the presumptive axial endoderm is already lying deep within the egg in this region. The floor of the blastocoel is markedly small-celled before gastrulation starts, and it appears likely that some of these cells become directly incorporated into the endoderm, but their fate cannot as yet be certainly decided.

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